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(54) Title: BREAST CANCER RESISTANCE PROTEIN (BCRP) AND THE DNA WHICH ENCODES IT

(57) Abstract

The Breast Cancer Resistance Protein is described, as well as the cDNA encoding said protein. This protein has been found to confer resistance to cancer chemotherapeutic drugs.

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Breast Cancer Resistance Protein (BCRP) and the DNA Which Encodes It

This application is based upon U.S. Provisional 60/073763, filed 2/5/98.

Field of the Invention

The invention relates to the family of proteins known as multidrug resistance proteins. These proteins are xenobiotic transporters which confer resistance to cancer chemotherapeutic drugs. The invention describes a new protein member of this family called Breast Cancer Resistance Protein (BCRP) and the DNA which encodes it.

Background of the Invention

The development of resistance to multiple chemotherapeutic drugs frequently occurs during the treatment of cancer. Two transmembrane xenobiotic transporter proteins, P-glycoprotein (Pgp) and the multidrug resistance protein (MRP) are capable of causing multidrug resistance when transfected into drug-sensitive cells in culture (1,2). Despite this, the role that these transporters play in clinical drug resistance exhibited by human cancers is unclear, and alternate or additional drug resistance mechanisms operative in this disease have been sought.

To address this problem, Chen et. al. (3) selected human breast carcinoma MCF-7 cells for resistance to the anthracycline doxorubicinin the presence of verapamil, an inhibitor of Pgp. The resultant multidrug resistant subline, MCF-7/AdrVp, exhibits marked

Summary of the Invention

The discovery described in the instant invention fulfills the above needs. The discovery of the BCRP and its corresponding gene greatly advance the knowledge in the art of the drug resistance mechanism by providing a novel xenobiotic transporter which is overexpressed in a variety of drug-resistant human cancer cell lines, and confers resistance to many chemotherapeutic agents.

BCRP is an about 655 amino acid protein and is encoded by a gene which has about 2418 nucleotide cDNA. The protein demonstrates activity and has a sequence homology which places it in the ATP-binding cassette (ABC) superfamily of transporter proteins. The molecular mass is approximately 72.3 kilodaltons (kD) exclusive of any glycoylation. Expression of BCRP in drug-sensitive human cancer cells confers resistance to mitoxantrone, doxorubicin, and daunorubicin, and reduces daunorubicin accumulation in the cloned transfected cells.

It is an object of the present invention to provide a mammalian protein that is a multi-drug resistant (MDR) protein and a xenobiotic transporter, and is called Breast Cancer Resistance Protein (BCRP).

It is also an object of the present invention is to provide the gene and/or cDNA which encodes said mammalian MDR protein.

It is another object of the invention to provide antisense

fragments of the BCRP gene which inhibit the expression of the BCRP *in vivo*.

Yet another object of the present invention is to provide a method of using probes derived from the BCRP gene as a diagnostic tool to quantify gene expression or gene amplification in specimens taken from patients with cancer.

It is another object of the invention to provide antibodies to the BCRP.

It is yet another object of the invention to provide a method of reversing the drug resistance of the cancer cells by administering BCRP antibodies.

It is yet another object of the invention to provide a method of reversing the drug resistance of the cancer cells by administering Fumitremorgin C.

It is another object of the invention to provide a method of enhancing a patient's chemotherapy treatment for breast cancer by administering antibodies to the patient to inhibit the resistance-activity of BCRP.

These and other objects of the present invention, which will be apparent from the detailed description of the invention provided hereinafter, have been met, in one embodiment, by substantially pure BCRP and the gene encoding BCRP.

Brief Description of the Drawings

Figure 1A is an autogradiograph of the RNA fingerprinting of MCF-7 cells.

Figure 1B is an autoradiograph of a Northern blot hybridization of mRNA from MCF-7/W, MCF-7/AdrVp, and MCF-7/AdrVpPR cells.

Figure 1C is an autoradiograph of a genomic Southern blot hybridization of DNA from MCF-7/AdrVp, MCF-7/W and MCF-7/AdrVpPR cells.

Figure 2A is the deduced amino acid sequence of BCRP with motifs.

Figure 2B shows the relative similarity of BCRP to selected members of the ABC transporter superfamily.

Figure 2C is the cDNA sequence which encodes the BCRP.

Figure 2D is a graph of a phylogram showing the evolution of the amino acid seqeunce of BCRP in relation to certain other members of the ABC family of transport proteins.

Figure 3 shows an autoradiograph of a multiple tissue Northern blot.

Figure 4A is an autoradiograph of a Northern blot of subclones of BCRP transfectants.

Figure 4B is a graph of Daunorubicin (DNR) accumulation and

retention in the pcDNA3 vector control cells and BCRP-transfected clones 6 and 8.

Figure 4C shows the relative resistance factors-MCF-7, vector control, clones 19, 6, and 8.

Figure 4D are graphs showing the effect of various chemotherapeutic drugs' concentrations on BCRP-transfected MCF-7 clone 8 cell survival.

Figure 4E shows a graph of the effects of ATP deletion of the retention of rhodamine 123 by transfectant MCF-7/pcDNA3 (empty vector control) or MCF-7/BCRP clone 8 cells.

Figure 5 is a table showing the effect of various chemotherapeutic drugs on BCRP-transfected MCF-7 cells.

Figure 6 is an autoradiograph showing the expression of Human *w* gene in MCF-7 cells detected by the Reverse Transcription-Polymerase chain reaction (RT-PCR).

Figure 7 is an autoradiograph showing the expression of BCRP in samples of blast cells from patients with acute myelogenous leukemia.

Figure 8A, 8B, and 8C are autoradiographs showing the results of Northern blot hybridizations of mRNA from various drug resistant cell lines probed with a BCRP probe.

Figure 9 is an autoradiograph of a Southern blot hybridization

from various MCF-7 cell lines.

Figure 10 is a graph showing the results of administration of FTC to BCRP transfected cells.

Detailed Description of the Invention

A novel gene and the protein encoded by said gene, called the Breast Cancer Resistance-associated Protein (BCRP) are described in the instant invention. The BCRP is shown to be overexpressed in human multi-drug resistant (MDR) breast carcinoma cells, colon carcinoma, gastric carcinoma, fibrosarcoma, and myeloma origin. The BCRP is a xenobiotic transporter which confers resistance to multiple chemotherapeutic drugs, and belongs to the ABC transporter superfamily. The BCRP appears to be responsible for the alteration in drug transport and drug resistance manifested by various cancer cells.

The present invention pertains partially to the BCRP, to fragments of this factor, as well as to functional derivatives, agonists and antagonists, and metabolic breakdown products of this factor. The BCRP amino acid sequence is depicted in SEQ ID No. 1 and Figure 2A. The invention especially concerns agents which are capable of inhibiting BCRP, preferably antibodies to BCRP or antisense probes to the BCRP gene. The invention further encompasses chemical agents which inhibit expression of the BCRP gene or mRNA, including

Fumitremorgin C (FTC). The invention also concerns methods of inhibiting activity of BCRP or expression of the BCRP gene by administering such agents.

A "functional derivative" of BCRP is a compound which possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of BCRP. The term "functional derivatives" is intended to include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. A "fragment" of a molecule such as BCRP, is meant to refer to any polypeptide subset of the molecule. A functional fragment means that a molecule with a similar, but not identical, amino acid sequence, but has the same function of the full length BCRP. A "variant" of a molecule such as BCRP is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof. A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity.

Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules is not found in the other, or if the sequence of amino acid residues is not identical. An "analogue" or agent which mimics the function of a molecule such as BCRP is meant

to refer to a molecule substantially similar in function but not in structure to either the entire molecule or to a fragment thereof. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980). Procedures for coupling such moieties to a molecule are well known in the art. More specifically, the scope of the present invention is intended to include functional derivatives of BCRP which lack one, two, or more amino acid residues, or which contain altered amino acid residues, so long as such derivatives exhibit the capacity to influence cell resistance to chemotherapy.

An "antagonist" of BCRP is a compound which inhibits the function of BCRP. Such antagonists can be immunoglobulins (such as, for example, monoclonal or polyclonal antibody, or active fragments of such antibody). The antagonists of the present invention may also include non-immunoglobulin compounds (such as polypeptides, organic compounds, etc.), and substrates of BCRP transport that may modulate or inhibit the transport of cytotoxic drugs. Antagonists, or

inhibitors of BCRP are one embodiment of the invention. These antagonists or inhibitors are useful for inhibiting the drug resistance effect caused by BCRP on cancer cells. The preferred inhibitor is an antibody raised to the BCRP, an antigenic fragment thereof, or a drug which blocks BCRP transporter activity. A preferred inhibitor which is a drug is fumitremorgin C (FTC), a mycotoxin. FTC was obtained from Dr. Lee Greenberg at Wyeth-Ayerst Laboratories in Pearl River, New York.

A polyclonal antibody capable of binding to BCRP can be prepared by immunizing a mammal with a preparation of BCRP or functional derivative of BCRP. Methods for accomplishing such immunizations are well known in the art. Monoclonal antibodies or fragments thereof can also be employed to assay for the presence or amount or BCRP in a particular biological sample. Such antibodies can be produced by immunizing splenocytes with activated BCRP (7). The BCRP-binding antibodies of the present invention can be administered to patients to reduce resistance to chemotherapy drugs, and hence enhance their treatment. Methods of administration will depend on the particular circumstances of each individual patient and are within the skill of those skilled in the art.

The BCRP of the present invention may be obtained by natural processes (such as, for example, by inducing the production of BCRP

from a human or animal cell); by synthetic methods (such as, for example, by using the Merrifield method for synthesizing polypeptides to synthesize BCRP, functional derivatives of BCRP, or agonists or antagonists of BCRP (either immunoglobulin or non-immunoglobulin); or by the application of recombinant technology (such as, for example, to produce the BCRP of the present invention in diverse hosts, e.g., yeast, bacterial, fungi, cultured mammalian cells, to name a few, or from recombinant plasmids or viral vectors). The compounds of the present invention are said to be "substantially free of natural contaminants" if preparations which contain them are substantially free of materials with which these products are normally and naturally found.

The choice of which method to employ will depend upon factors such as convenience, desired yield, etc. It is not necessary to employ only one of the above-described methods, processes, or technologies to produce BCRP; the above-described processes, methods, and technologies may be combined in order to obtain BCRP. It is most preferable to prepare BCRP by expressing the gene or cDNA sequence which encodes the BCRP protein. Such gene or cDNA sequence hereinafter termed the "BCRP gene" or "BCRP cDNA sequence".

The technique of RNA fingerprinting was employed to clone the BCRP cDNA. RNA fingerprinting uses the polymerase chain

reaction (PCR) and degenerate primer pairs to amplify cellular mRNA. This technique is based on modifications of the technique of "Differential Display of mRNA" developed by Liang and Pardee (6). We used these techniques as a means to discover genes that are differentially expressed in drug-selected cell lines compared to parental cells. The major difference between RNA Fingerprinting and Differential Display is that the mRNA fingerprinting protocol uses a single cDNA synthesis reaction, followed by amplification with upstream and downstream primers. Differential Display uses 9 to 12 cDNA syntheses for each RNA sample with an anchored oligo(dT) primer, followed by amplification with an upstream primer.

The cloned BCRP gene, obtained through the methods described above and in the examples, may be operably linked to an expression vector, and introduced into bacterial, or eukaryotic cells to produce BCRP protein. Techniques for such manipulations are disclosed in Maniatis, T. *et al.* supra, and are well known in the art (8).

The BCRP cDNA sequence is about 2418 nucleotides long. The BCRP cDNA is depicted in SEQ ID No. 2 or Figure 2C. The BCRP cDNA can be used to express the BCRP. Also, the BCRP cDNA sequence, or a portion thereof, can be used as a probe in a Northern blot assay or for selection of probes in a RT-PCR assay to measure BCRP mRNA in various tissue samples. Measurement of expression of BCRP by

Northern blot or RT-PCR assay can be determinative of drug response to chemotherapeutic drugs over time. The techniques for these assays are described in the examples and are well-known in the art (8). Therefore, such an assay could be used to determine if a patient's failure to respond to chemotherapy is due to overexpression of BCRP, and hence resistance to the drugs. Also, antisense probes could be developed based on the cDNA sequence depicted in SEQ ID 2 and figure 2C. These probes can be administered to patients to bind to the BCRP cDNA endogenously and hence inhibit the expression of the BCRP. Such a therapy could be used to halt or slow a patient's propensity to become resistant to the chemotherapy drugs and hence render treatment more effective. Techniques for the production and administration of antisense probes are well known in the art. Techniques of nucleic acid hybridization and cloning are well known in the art (8).

The data presented in the examples and corresponding figures strongly support the conclusion that the novel ABC family member BCRP reported here is a xenobiotic transporter that is primarily responsible for the drug resistance phenotype of MCF-7/AdrVp cells.

The overexpression of BCRP in several cancer cell lines is also shown in the present invention. These cell lines include colon carcinoma cells S1, HT29, gastric carcinoma cells EPG85-257,

fibrosarcoma cells EPR86-079, and myeloma 8226 cells. The overexpression of BCRP mRNA in each of these cell lines, and the amplification of the BCRP gene in the drug-resistant cells demonstrate an important role for BCRP in resistance to cytotoxic agents. Furthermore, the enforced overexpression of BCRP in MCF-7 cells diminished daunorubicin cellular accumulation and imparted a pattern of drug cross-resistance to the transfected cells that was virtually identical to that of MCF-7/AdrVp cells. The degree of overexpression of BCRP in transfectant clones 6 and 8 correlates with the alterations in the intracellular steady state level of daunorubicin and their degree of resistance to mitoxantrone, daunorubicin and doxorubicin.

A major difference between the BCRP-overexpressing transfectant clones and the original MCF-7/AdrVp subline is that the degree of drug resistance in the latter is greater than in the transfected cells, while the steady state BCRP mRNA levels in each are comparable (Figure 4A). A number of possibilities may contribute to this difference. Differences in protein stability and/or localization may contribute to the full drug-resistant phenotype, or the expression of other proteins may be required. Recently, we reported that members of the carcinoembryonic antigen (CEA) family, primarily the non-specific cross reacting antigen (NCA) and CEA itself, are markedly

overexpressed on the cell surface of MCF-7/AdrVp and MCF-7/AdrVpPR cells compared to drug-sensitive MCF-7 cells (15). A high density of these acidic glycoproteins on the cell surface may protonate drugs such as mitoxantrone, daunorubicin or doxorubicin which prevents entry into the cell. Indeed, Kawaharata, *et.al.* (16) reported that the enforced expression of CEA in transfected NIH3T3 cells results in both diminished accumulation of and resistance to doxorubicin in the transfected cells. Hence, the relative overexpression of CEA family members on the MCF-7/AdrVp cell surface could act in concert with BCRP to cause greater resistance to mitoxantrone, doxorubicin and daunorubicin than that caused by BCRP alone. This hypothesis could be tested by co-transfecting the MCF-7/BCRP-clone 8 subline with an expression vector containing NCA or CEA.

Another possible explanation for the greater degree of resistance of MCF-7/AdrVp cells compared to the transfectants is that BCRP is part of a multiprotein transporter complex. The translocation pathway of typical ABC transporters consists of two ATP-binding domains and two highly hydrophobic domains which contain membrane-spanning regions. This can be accomplished in a single molecule, as is the case of MRP or Pgp, which are twice the size of BCRP (approximately 1,300 compared to 655 amino acids). Alternatively, the active complex of certain ABC transporters can be formed by the heterodimerization of

two non-identical proteins, each of which contains a single ATP-binding and hydrophobic region. The *w* and brown (*b*) proteins of *Drosophila* and the Tap-1 and Tap-2 proteins that transport major histocompatibility class I proteins are examples of ABC family members that exhibit such a cooperative interaction. The presence of the phosphopantetheine attachment site on BCRP suggests that BCRP may be a part of a multiprotein complex. Thus, it is possible that BCRP has a protein cofactor(s) which makes it a much more efficient transporter in a heteromeric state. The activation or overexpression of this cofactor in MCF-7/AdrVp relative to MCF-7 cells could explain the increased drug transport in the MCF-7/AdrVp subline relative to the BCRP transfectants.

The finding of elevated expression of BCRP mRNA in the human colon carcinoma S1M1-3.2 cells suggests that BCRP is the "non-Pgp, non-MRP" drug transporter manifested by this multidrug-resistant cell line. This is of particular importance because of the recent report (25) of a specific inhibitor of the transporter identified in S1M1-3.2 cells. This inhibitor, fumitrimorgin C (FTC), does not reverse resistance in cells that overexpress Pgp or MRP. Figure 10 shows that FTC is able to enhance the accumulation and inhibit the efflux of BBR 3390 (an aza-anthrapyrazole drug that is effluxed by BCRP) in BCRP-transfected MCF-7 cells.

The following examples are provided for illustrative purposes only and are in no way intended to limit the scope of the present invention. All references cited are incorporated by reference.

Examples

Cell lines. MCF-7 breast carcinoma cells, their drug- resistant subline MCF-7/AdrVp, and a partially drug-sensitive revertant subline (MCF-7/AdrVpPR, obtained from Dr. Antonio Fojo, Medicine Branch, National Cancer Institute), were maintained in culture as described previously (5). The MCF-7/AdrVp subline was continuously maintained in the presence of 100 ng/ml doxorubicin (Pharmacia Adria, Dublin, OH) and 5 μg/ml verapamil (Sigma Chemicals, St. Louis, MO).

Growth conditions for the cell lines used in the Northern blot studies are contained in the references listed in Table 1. The S1M1-3.2 colon carcinoma cells were derived from S1 cells (a subclone of human colon carcinoma cell line LS174T) by selection for growth in increasing concentrations of mitoxantrone until a final concentration of 3.2 μM was acheived. HL-60/MX2 cells were purchased from the American Type Culture Collection (Manassas, VA), and maintained in culture as described previously (17).

Example 1: Synthesis of cDNA by reverse transcription of mRNA

Purified total cellular RNA (2 μg) from MCF-7/W, MCF-7/AdrVp or MCF-7/AdrVpPR cells which have partially reverted to drug sensitivity by culture in the absence of the selecting agents were reverse transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase in the presence of an oligo(dT) primer (0.1 μM), and 1 mM dNTP at 42°C for 1 hour. The reactions were terminated by heating at 75°C for 10 minutes. The cDNAs thus produced were stored at -20°C until further use.

Example 2 RNA Fingerprinting

RNA fingerprinting was performed using the Delta™ RNA fingerprinting kit (Clontech Laboratories, Palo Alto, CA), with minor modifications. RNA fingerprinting is accomplished by amplification of the cDNA by the polymerase chain reaction (PCR), using random primers.

For each fingerprinting reaction, cDNA diluted 1:10 (dilution A) or 1:40 (dilution B) from each cell line was amplified with one upstream (P) and one downstream (T) primer in the presence of 50 μM dNTP, 50 nM [^{33}P]dATP, and the "Advantage KlenTaq Polymerase Mix" supplied with the Clontech kit. The upstream P primers were

arbitrary 25-mers. The downstream T primers were 30-mer anchored oligo(dT)primers whose 3' terminal contained the sequence 5'-$T_9N_1N_1$-3', where N_1 is A, C or G. The P primer binds to the cDNA based on chance homology. We paired ten P primers and nine T primers to give 90 possible combinations.

The first three PCR cycles were performed at a relatively low stringency (annealing temperature 40°C). Because of this, the P primer bound imperfectly, which increased the number of amplified products. The products of these early cycles were then amplified by 24 PCR cycles at high stringency (annealing temperature 60°C). Control PCR reactions were prepared containing sterile water instead of cDNA (water control), or 0.02 μg of total cellular RNA (RNA control). The RNA controls were prepared to assess whether the RNA was contaminated with genomic DNA.

Following the PCR reaction, a small amount of each reaction mixture was loaded onto a 5% polyacrylamide gel, after which the gels were dried, then autoradiographs made (Figure 1A). These autoradiographs demonstrated a characteristic "RNA Fingerprint" pattern of 50 to 100 PCR product bands of 100 to 2000 nucleotides in length. Lanes 1, 3, and 5 are reaction mixes where cDNA diluted 1:10 (dilution A) was added; lanes 2, 4, and 6 represent reaction mixtures where cDNA diluted 1:40 (dilution B) was added. Lanes 7 and 8 are

"H_2O controls", where sterile water was added to the PCR reaction mixture instead of cDNA. Lanes 9, 10 and 11 are "RNA controls", where 0.02 μg of cellular RNA from MCF-7/W, MCF- 7/AdrVp, or MCF-7/AdrVpPR cellular is added instead of cDNA. These "RNA controls" serve to indicate contamination of the RNA with genomic DNA. The autoradiographs were inspected for PCR products that were produced in greater abundance in reactions that used reverse transcribed RNA from MCF-7/AdrVp cells, compared to those that used RNA from MCF-7/W or MCF-7/AdrVpPR cells (Figure 1A). The **ARROW** indicates a PCR product that represents a mRNA species that is overexpressed in MCF-7/AdrVp cells, compared to MCF-7/W or MCF-7/AdrVpPR cells. This is the PCR product that was cut out of the gel and amplified and cloned using the "TA Cloning" method, the desired clone of which was called Clone 8 (see below).

Example 3: Amplification of the target cDNA by TA cloning

The PCR product overexpressed in MCF-7/AdrVp cells was excised from the dried gel and eluted by boiling in 40 ml ddH_2O for 5 min, then amplified by PCR for 20 cycles using the original primers and separated on 2% agarose/ethidium bromide gels. These PCR products were then ligated into a "TA Cloning Vector" plasmid, pCR®2.1, which was then cloned using standard techniques for PCR products (Original

TA Cloning® Kit, Invitrogen Corporation, San Diego, CA).

The pCR®2.1 plasmids containing the PCR product were used to transform the TOP 10F strain of *E. coli*. Individual bacterial colonies were picked and plasmid DNA was isolated by minipreps (Wizard™ Miniprep, Promega, Madison, WI). Plasmid DNA was amplified by PCR with the original "P" and "T" primers, then subjected to gel electrophoresis. The original sized band was cut out, and the DNA was isolated by boiling in 100 μl ddH_2O at 100°C for 5 min. An aliquot of the DNA was reamplified by PCR with the original primers for 20 cycles. A single band was visualized on ethidium bromide gels which was cut out, electroeluted then precipitated.

Example 4 Isolation of the BCRP clone

The "reverse" Northern blot method was used to screen the TA vector clones. Briefly, a "reverse" Northern analysis was performed as follows. The PCR product isolated from 12 different colonies of *E. coli* that was transformed by the pCR2.1 plasmid were fixed in duplicate to Zeta Probe (BioRad, Richmond, CA) membranes in a slot blot apparatus. One of the duplicate membranes was probed with the [^{33}P]-labeled PCR reaction mixture that amplified MCF-7 cDNA using

the original "P" and "T" primers in the RNA Fingerprinting kit. The other membrane was probed with the original [^{33}P]-labeled parallel PCR reaction mixture that amplified the cDNA produced from MCF-7/AdrVp cells, using standard Northern blot conditions of hybridization, after which the binding of probe was assessed by autoradiography. A single TA clone (Clone 8 - SEQ ID No. 7) was thus identified whose PCR product insert identified a 2.4 kb mRNA species that was markedly overexpressed in MCF-7/AdrVp cells, compared to MCF-7 cells (Figure 1B, top panel). The partially revertant MCF-7/AdrVpPR subline had intermediate expression of the 2.4 kb mRNA species (Figure 1B, top panel). To control for equivalence in lane loading, the blot was stripped then reprobed with radiolabeled 18S RNA (Figure 1B, bottom panel).

Southern blots were performed using the Clone-8 PCR product. Briefly, DNA was isolated, digested with *Eco*R1, subjected to agarose gel electrophoresis, transferred and fixed to a nitrocellulose filter. The filter was probed with the Clone-8 PCR product that was end-labeled with [^{32}P]-dCTP, then the radioautograph shown was made (Figure 1C, top panel). This demonstrated that the cognate gene for BCRP was amplified in both MCF-7/AdrVp and MCF-7/AdrVpPR cells, compared to parental MCF-7 cells (Figure 1C, top panel). The lower panel in Figure 1C shows the ethidium bromide-stained agarose gel

electrophoretogram of the corresponding genomic DNA after digestion with *Eco*R1, to demonstrate approximate equivalence of gel loading.

Example 5 Sequencing of the BCRP clone

Sequencing of the cDNAs was performed with an automated DNA sequencer (Perkin Elmer, Inc., Foster City, CA). All DNA sequences were confirmed by sequencing in the reverse direction. The differentially expressed PCR product in the TA Clone 8 was sequenced and found to be a 795 bp cDNA (SEQ ID No. 7). Protein database searches of the deduced amino acid sequence revealed a high degree of homology to members of the ABC superfamily of transporter proteins.

Example 6 Isolation of the full-length BCRP cDNA

An MCF-7/AdrVp cDNA library was constructed using the CapFinder™ PCR cDNA library construction kit (Clontech) according to the manufacturer's protocol. The CapFinder™ technique is designed specifically to produce full-length double stranded cDNA. The 795 bp Clone 8 cDNA fragment was radiolabeled and used as a probe to screen the cDNA library prepared from MCF-7/AdrVp cells. Positive clones isolated were subjected to secondary and tertiary screening, then tested by Northern blot hybridization using RNA obtained from MCF-7, MCF-7/AdrVp and MCF-7/AdrVpPR cells.

Multiple clones were found to have 2.4 kb inserts, the approximate size of the BCRP mRNA suggested by Northern blotting.

Four of the 2.4 kb inserts were ligated into the pCR2.1 plasmid, then these TA vectors were cloned in *E. coli* (as described above). One TA vector clone containing a 2.4 kb cDNA fragment insert was identified and isolated. Sequencing of the 2.4 kb cDNA insert was performed with an automated DNA sequencer (Perkin Elmer Inc., Foster City, CA). All DNA sequences were confirmed by sequencing in the reverse direction. After sequencing, the cDNA insert was found to be 2418 bp in length as in Figure 2C or SEQ ID No. 2. Analysis of the cDNA for open reading frames (ORF) using the program "FRAMES" contained in the Genetics Computer Group (GCG) software package indicated the presence of a long ORF that began at position 239, and ended with the stop codon TAA at position 2204-6. The deduced amino acid sequence of this ORF is shown in Figure 2A, and SEQ ID No. 1. The protein has 655 amino acids and a approximate molecular weight of about 72.3 kilodaltons. The protein encoded by this sequence has been designated Breast Cancer Resistance Protein, or BCRP (Figure 2A).

Analysis of the sequence of BCRP with the GCG program "MOTIFS" demonstrated a single Walker "A" ATP/GTP binding region (11) at amino acids 80-87 and a phosphopantetheine attachment

site at amino acids 213-228 (Figure 2A). Phosphopantetheine (or pantetheine 4' phosphate) is the prosthetic group of acyl carrier proteins in some multienzyme complexes where it serves in the attachment of activated fatty acid and amino-acid groups (12).

Examination of BCRP structure with GCG programs "PEPPLOT" and "PLOTSTRUCTURE" revealed a relatively hydrophilic amino-terminal domain (amino acids 1-400) that contains the ATP-binding sequence and a relatively hydrophobic carboxy-terminal domain (amino acids 401-655), containing at least three putative transmembrane domains (TM1, TM2, and TM3), and four potential N-glycosylation sites (Glyc) (Figure 2A). The transmembrane domains were estimated by the use of a program to predict helices in integral membrane proteins (13). Analysis of the BCRP sequence by the GCG program "DOTPLOT" demonstrates that the peptide is homologous with one-half of the duplicated Pgp or MRP molecule, except that Pgp or MRP have the configuration NH_2-[transmembrane domains]-[ATP binding 1]- [transmembrane domains]-[ATP binding 2]-COOH, whereas that of BCRP is NH_2-[ATP binding]-[transmembrane domains]-COOH.

The relative similarity of BCRP to other members of the ABC transporter superfamily was determined using the "PILEUP" program of GCG. This analysis demonstrated that the peptide sequence of BCRP is only distantly related to P-glycoprotein (PgP or Mdr1) or MRP (Figure

2B).

Example 7 Comparison of BCRP sequence to the *w* sequence

Analyses of cDNA and deduced protein sequences were accomplished using protein and nucleotide sequence databases that were accessed using the Wisconsin Sequence Analysis Package Version 8 (Genetics Computer Group [GCG], Madison, WI) which are available through the Frederick Cancer Research Center's Supercomputing Facility (Frederick, MD).

A "FASTA" comparison of the BCRP amino acid sequence revealed a high degree of homology to at least 50 ATP-binding cassette transport proteins. The highest match was PIR2:G02068, the human homologue of the *Drosophila* white (*w*) gene, which has 638 amino acids, and is 29.3% identical to BCRP. The *w* gene in *Drosophila* functions in the cellular transport of guanine and tryptophan, which are retinal pigment precursors (9). We found that the human homologue of *w* is not overexpressed in MCF-7/AdrVp cells compared to MCF-7 cells, as detected by a reverse-transcription PCR assay (Figure 6).

The program "Oligo" (Version 5.0, National Biosciences, Inc., Plymouth, MN) was used to help determine suitable primers for detection of the human homologue of *w* by reverse transcription-PCR.

These assays were done using a modification of those described previously for beta actin and MRP (10), except that primers specific for the *w* gene were used instead of MRP. The upper primer began at 5' position 2136 of human *w* mRNA, and had the sequence 5'-CGA CCG ACG ACA CAG A-3) (SEQ ID No. 3); The lower primer began at 3' position 2590, and had the sequence 5'-CTT AAA ATG AAT GCG ATT GAT-3') (SEQ ID No. 4). To assure uniformity of gel loading, a reverse transcription-PCR assay for beta-actin was also performed. The final concentrations of primers used was 200 nM. Twenty-five cycles of denaturation (94°C, 1 minute), annealing (50°C, 1 minute) and elongation (72°C, 2 minutes) were carried out. Figure 6 shows an agarose gel electrophoresis of an aliquot of the PCR reaction mixtures that used RNA from MCF-7 or MCF-7/AdrVp cells demonstrating that both human *w* and beta-actin are expressed approximately equally in these cell lines.

Example 8: Northern blots of various human tissue with BCRP probe (Clone 8)

Northern blotting with a ^{32}P-labeled Clone 8 cDNA probe was performed. Pre-blotted agarose gel-electrophoresed RNA from multiple tissues was purchased from Clontech, for use in multiple tissue Northern blot assays (Figure 3). The greatest expression of BCRP

was seen in placental tissue, with lower amounts of expression demonstrable in brain, prostate, small intestine, testis, ovary, colon and liver. BCRP transcripts were below the level of detection in heart, lung, skeletal muscle, kidney, pancreas, spleen, thymus and peripheral blood leukocytes.

Example 9: Expression of BCRP in MCF-7 cells--Functional Studies

The full-length BCRP cDNA was inserted into the multiple cloning site of expression vector pcDNA3 (Invitrogen). Following subcloning of the pcDNA3-BCRP construct, DNA sequence analysis was performed to confirm that the insert in the clone that was chosen was in a sense orientation to the CMV promoter of the pcDNA3 vector. MCF-7 cells were transfected with pcDNA3-BCRP, using the calcium phosphate precipitation method (17), selected by culture with geneticin (G418, 1 mg/ml), then subcloned by limiting dilution in 96 well flat-bottomed culture plates. Subclones were tested for expression of BCRP mRNA by Northern blot analysis, using radiolabeled Clone 8 cDNA as a probe (Figure 4A). As a control, MCF-7 cells were also transfected with the empty pcDNA3 vector, then selected by growth in medium containing 1 mg/ml G418 (Figure 4A). Two clones of MCF-7 cells transfected with pcDNA3-BCRP that were found to overexpress

BCRP (clones 6 and 8) were selected and expanded for further studies (Figure 4A). A third clone of pcDNA3-BCRP transfected cells, clone 19, did not overexpress BCRP, and was selected for study as a control.

Example 10: Effect of Chemotherapeutic Drugs on BCRP-transfected MCF-7 cells

Daunorubicin accumulation and retention was examined in the transfected cells by means of flow cytometry. The BCRP-overexpressing clones 6 and 8 displayed diminished accumulation and retention of daunorubicin, compared to the vector-transfected controls (Figure 4B), with intracellular steady-state concentrations of drug in clones 8 and 6 respectively approximately 30% or 50% of that attained in the vector control cells. This difference was not due to differences in cell volume, since the volumes of the BCRP-overexpressing sublines tested was not less than that of the empty vector-transfected control cells. The cell volumes, measured by Coulter Channelyzer^{TM} are 2515$\pm$56, 3074$\pm$112 and 2459$\pm$56 um^3 for MCF- 7/BCRP-clone 6, MCF-7/BCRP-clone 8 and MCF-7/pcDNA3 vector control cells, respectively. These values are comparable to our previous measurements of MCF-7 cell volumes (5).

The sensitivities of the various transfected sublines to chemotherapeutic agents were tested by the sulforhodamine-B (SRB) cytotoxicity assay (14). The LC_{50}, defined as the concentration of drug

that caused lethality to 50% of the cells, was calculated. From this, the "Fold of Resistance" (RF) was calculated by dividing the LC_{50} for a given drug against a transfected cell line by the LC_{50} of that drug against non- transfected MCF-7 cells. The BCRP-overexpressing clones 6 and 8 displayed resistance to mitoxantrone, daunorubicin and doxorubicin, compared to non-BCRP-overexpressing clone 19 cells, MCF-7 cells, or the empty vector-transfected controls (Figures 4C, 4D, 5). Figure 5 contains the median LC_{50} values for multiple cytotoxicity experiments for all cell lines and drugs tested. Figure 4D shows typical LC_{50} studies for the six drugs tested for MCF- 7/W and MCF-7/pcDNA3-BCRP clone8 cells to illustrate the data from which the LC_{50} values were derived, and the accuracy of the measurements. The asterisk and solid line in Figure 4D indicate MCF-7/W cells, the closed squares and dotted lines represent MCF-7/pcDNA3-BCRP clone 8 cells. The vertical bars in the figure represent the standard deviation of six replicate determinations.

Like MCF-7/AdrVp cells, the MCF-7/BCRP transfectant clones 6 and 8 displayed the greatest degree of resistance to mitoxantrone. The pattern of cross-resistance displayed by the BCRP-overexpressing transfected cells is very similar to that displayed by MCF-7/AdrVp cells, except that MCF-7/AdrVp cells have greater relative resistance to all

cytotoxic drugs within the phenotype. The BCRP-transfected clones 6 and 8 remained relatively sensitive to idarubicin, cisplatin and paclitaxel (taxol), as are MCF-7/AdrVp cells (Figures 4C, 4D and 5).

To determine the effects of ATP depletion on the retention of rhodamine 123 by the BCRP transfected cells compared to controls, cells were incubated in complete medium or under ATP-depleting conditions. MCF-7 cells were depleted of ATP by incubation in glucose-free DMEM containing 50mM 2-deoxy-D glucose and 15mM sodium azide for 20 minutes (37°C). Rhodamine 123 was added (0.5 μg/ml final concentration) for an additional 30 minutes. The cells were placed on ice, washed free of rhodamine, and incubated under ATP-depleting conditions for an additional 30 minutes, and rhodamine retention was determined by flow cytometry (excitation 488nm, emission 520nm). This demonstrates that the transport function of BCRP appears to depend on ATP.

Example 11: Expression of BCRP in blast cells from patients with acute myelogenous leukemia (AML) as detected by a reverse-transcription polymerase chain reaction (RT-PCR) assay.

The RT-PCR assays were performed using a modification of those described previously for beta actin and MRP (10), except that

primers specific for BCRP were used instead of MRP. For BCRP, the primers used were (sense) 5'-TTA GGA TTG AAG CCA AAG G-3' (SEQ ID No. 5), and (antisense) 5'-TAG GCA ATT GTG AGG AAA ATA-3' (SEQ ID No. 6). The 5' end of the sense primer begins at nucleotide position 1727 of the BCRP cDNA (SEQ ID No. 2 and Figure 2C); the 3' end of the antisense probe corresponds to position 2152 of the BCRP cDNA (Figure 2C). The final concentrations of primers used was 200 nM. The final magnesium concentration used for PCR was 700 uM. Thirty-five cycles of denaturation (94°C, 1 minute), annealing (50°C, 1 minute) and elongation (72°C, 2 minutes) were carried out. Following agarose gel electrophoresis of an aliquot of the PCR reaction mixture, the gels were transferred to nitrocellulose and Southern blotting was done as described previously (12), using the 795 bp Clone 8 PCR product (5' end labeled with ^{32}P-dCTP) as a probe for BCRP. The expected PCR product length is 446 bp.

Total cellular RNA was obtained from the blast cells of fourteen patients with AML. Controls were done using varying volumes of the PCR reaction mixture that was run with reverse-transcribed MCF-7/W RNA. The results of these controls and of the RT-PCR assays of the patient blast cell samples are depicted in Figure 7. These controls using MCF-7/W RNA indicate the RT-PCR assay we developed is quantitative. Note in Figure 7 that some patients have very low levels

of expression of BCRP, while others (patients 3, 4, 5 and 7) have levels of expression comparable to or greater than that of MCF-7/W cells. This variation in expression of BCRP amongst blast cell samples from AML patients holds open the possibility that those patients who have relatively high expression of BCRP are more resistant to treatment with the anti-neoplastic drugs which are susceptible to the resistance caused by BCRP (anthracyclines and mitoxantrone). Mitoxantrone and the anthracycline daunorubicin are important drugs used in the treatment of AML.

Example 12: Northern blot hybridization in various cancer cell lines.

Total cellular RNA was used for Northern analysis in all cases except for H209 or H69 cells, where poly A^+ RNA was used. RNA extraction and Northern blotting were performed by standard techniques, and as described in Example 4. A 795 bp fragment (clone 8, SEQ ID No. 7) of the 3' end of the 2418 bp BCRP cDNA was used as the hybridization probe after labeling with [^{32}P]-dCTP ("Prime-a-Gene" labeling kit, Promega, Madison, WI). To control for variations in sample loading, the blots were stripped, then re-hybridized with ^{32}P-labeled ß-actin or 18S RNA probes.

Figure 8A shows the results of the Northern blot hybidization of

mRNA from MCF-7 cells (lane 1), MCF-7/MITOX (lane 2), 8226/W cells (lane 3), and 8226/MR20 (lane 4). The blot was probed for BCRP with a 795-bp cDNA (Clone 8, SEQ ID No. 7) after labeling with ^{32}P-dCTP (top panel). To control for equivalence in sample loading, the blot was stripped and reprobed for ß-actin (bottom panel).

Figure 8B shows the results of a Northern blot hybridization of mRNA from S1/M1-3.2 cells (lane 1), S1/W cells (lane 2), MCF-7/W cells (lane 3), MCF-7/MX_{PR} cells(lane 4), MCF-7/MX_{RS250} cells (lane 5), MCF-7/MX_{RS600} cells (lane 6), MCF-7/VP (MRP+) cells (lane 7), MCF-7/Adr (Pgp+) cells(lane 8), MCF-7/MTX (DHFR+) cells (lane 9), MCF-7/AdrVp1000 (BCRP+) cells (lane 10). The blot was probed as described for figure 8A.

Figure 8C shows a Northern blot hybridization of mRNA from human colon carcinoma HT29 cells (lane 1), HT29RNOV cells (lane 2), human breast carcinoma MDA-MB-231 cells (lane 3), MDA-MB-231RNOV cells (lane 4), human fibrosarcoma EPF86-079 cells (lane 5), EPF86-079RNOV cells (lane 6), human gastric carcinoma EPG85-257 cells (lane 7), EPG85-257RNOV cells (lane 8), EPG85-257RDB (Pgp+) cells (lane 9), human pancreatic carcinoma EPP85-181 cells (lane 10), EPP85-181RNOV cells (lane 11), and EPP85-181RDB (Pgp+) cells (lane 12). The blots were probed as described above for figure 8A.

Example 13: Southern blot hybridization

Genomic DNA was isolated using standard techniques (8) from the parental drug sensitive MCF-7/W cells (lanes 1, 7), MCF-7/MX_{PR} cells (lanes 2, 8), MCF-7/MX_{RS250} cells (lanes 3, 9), MCF-7/MX_{RS600} cells (lanes 4, 10), MCF-7/VP cells (overexpress MRP, lanes 5, 11) and MCF-7/MTX cells (derive resistance by overexpression of DHFR, lanes 6, 12), digested with EcoR1 or BamH1, separated by 0.8% agarose gel electrophoresis, stained with ethidium bromide, transferred, and fixed to a nitrocellulose filter, using standard techniques (8). The filter was hybridized with the [^{32}P]-labeled 795 bp BCRP probe as described above for figure 8 (figure 9, top panel). Ethidium bromide stained 0.8% agarose gel electrophoresis of genomic DNA after digestion with the restriction endonucleases, and prior to nitrocellulose filter transfer, demonstrated approximate equivalency of sample loading (figure 9, bottom panel).

Example 14: Fumitremorgin C (FTC) effects on BCRP Transfected Cells

MCF-7 cells transfected with either the pcDNA3 empty vector or pcDNA3 containing the full-length BCRP cDNA (transfectant clone 8) were cultured as monolayers in tissue culture flasks. The effects of FTC on the accumulation of the aza-anthrapyrazole BBR3390 were

measured by exposing these cells to the fluorescent aza-anthrapyrazole BBR3390 (5 uM) in the presence or absence of 10 uM FTC for 60 minutes. Then, the cells were removed from the flasks by trypsinization, and intracellular BBR3390 content was measured by flow cytometry. The effects of FTC on BBR3390 retention were measured by exposing another set of cells (vector control and transfectant clone 8) to 5 uM BBR3390 with and without 10 uM FTC for 60 minutes, washing the cells free of drug, then reincubating the cells for an additional 30 minutes in fresh medium with and without FTC. Intracellular BBR3390 content was measured by flow cytometry. (See figure 10).

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We claim:

1) Breast Cancer Resistance Protein which induces resistance to cancer chemotherapeutic drugs, or fragments or derivatives thereof.

2) The protein of claim 1 which is about 655 amino acids in length.

3) The protein of claim 1 which has a molecular mass of 72.3 kilodaltons.

4) The protein of claim 1 which is substantially identical to the sequence in SEQ ID No. 1.

5) An antibody which binds to the protein of claim 1.

6) The antibody of claim 5 which is monoclonal.

7) The antibody of claim 5 which is polyclonal.

8) A gene which encodes the protein of claim 1.

9) The gene of claim 8 which is substantially identical to the sequence in SEQ ID No. 2.

10) An antisense probe which inhibits expression of the protein of claim 1.

11) The antisense probe of claim 10 which is substantially identical to the sequence in SEQ ID No. 7.

12) A method of determining the cause of a patient's resistance to cancer chemotherapy drugs by assaying for expression of the protein of claim 1, whereby overexpression of the said protein indicates that it is the cause.

13) A method of inhibiting the activity of the Breast Cancer Resistance Protein by administering the antibody of claim 5.

14) A method of inhibiting the activity of the Breast Cancer Resistance Protein by administering the antibody of claim 6.

15) A method of inhibiting the activity of the Breast Cancer Resistance Protein by administering the antibody of claim 7.

16) A method of inhibiting the activity of the Breast Cancer Resistance Protein by administering the probe of claim 10.

17) A method of inhibiting the activity of the Breast Cancer Resistance Protein by administering the probe of claim 11.

18) A method of enhancing a cancer patient's chemotherapy treatment by administering the antibody of claim 5.

19) A method of enhancing a cancer patient's chemotherapy treatment by administering the probe of claim 11.

20) A method of enhancing a cancer patient's chemotherapy treatment by administering Fumitremorgin C.

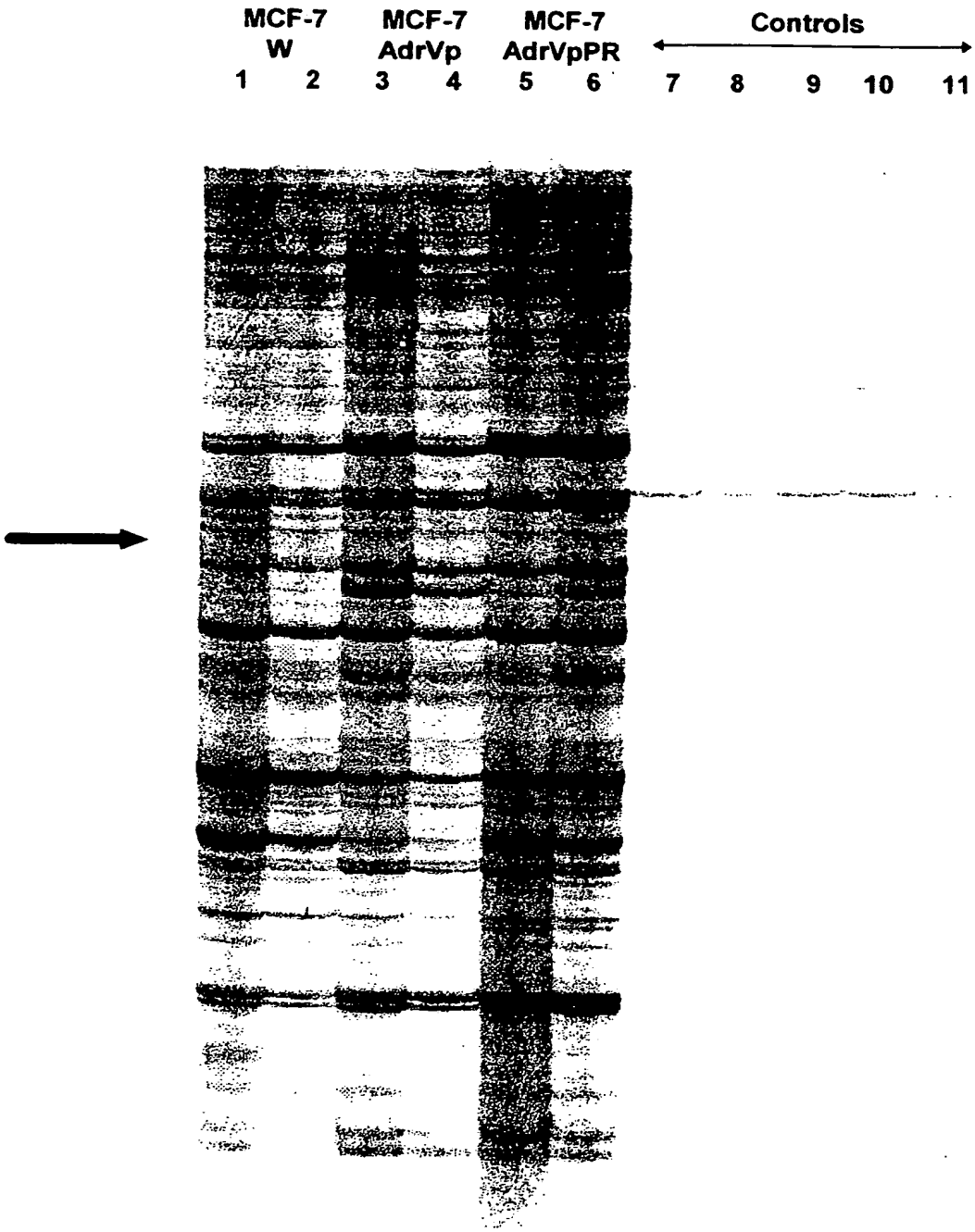

FIG. IA

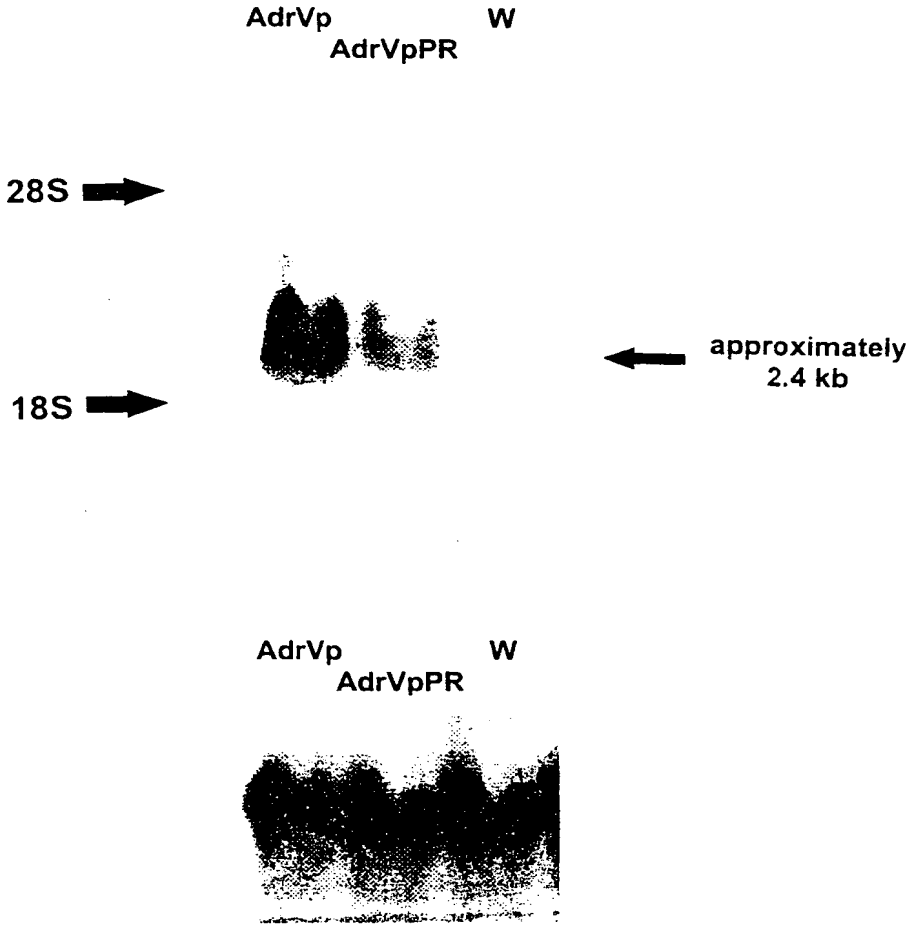

FIG. IB

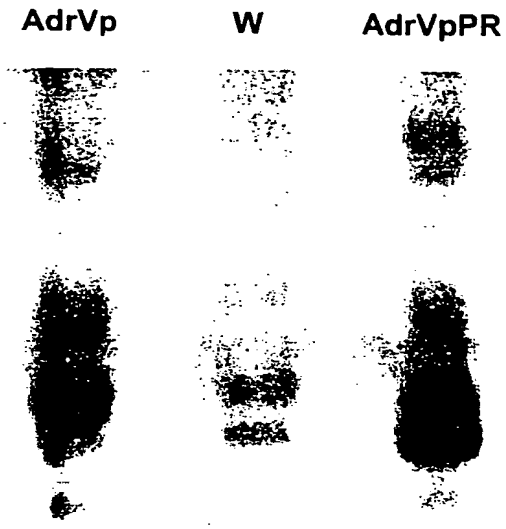

FIG. IC

Peptide Sequence of BCRP

Length: 663 amino acids

```
  1  AEKIKTLQMS SSNVEVFIPV SQGNTNGFPA TASNDLKAFT EGAVLSFHNI

                                          Walker A motif
 51  CYRVKLKSGF LPCRKPVEKE ILSNINGIMK PGLNAILGPT GGGKSSLLDV

101  LAARKDPSGL SGDVLINGAP RPANFKCNSG YVVQDDVVMG TLTVRENLQF

151  SAALRLATTM TNHEKNERIN RVIQELGLDK VADSKVGTQF IRGVSGGERK

                                Phosphopantetheine site
201  RTSIGMELIT DPSILFLDEP TTGLDSSTAN AVLLLLKRMS KQGRTIIFSI

251  HQPRYSIFKL FDSLTLLASG RLMFHGPAQE ALGYFESAGY HCEAYNNPAD

                                                    Glyc
301  FFLDIINGDS TAVALNREED FKATEIIEPS KQDKPLIEKL AEIYVNSSFY

351  KETKAELHQL SGGEKKKKIT VFKEISYTTS FCHQLRWVSK RSFKNLLGNP

                                        Glyc
401  QASIAQIIVT VVLGLVIGAI YFGLKNDSTG IQNRAGVLFF LTTNQCFSSV
     <------------------>
          TM 1

451  SAVELFVVEK KLFIHEYISG YYRVSSYFLG KLLSDLLPMT MLPSIIFTCI

501  VYFMLGLKPK ADAFFVMMFT LMMVAYSASS MALAIAAGQS VVSVATLLMT
                                                    <------

                                  Glyc
551  ICFVFMMIFS GLLVNLTTIA SWLSWLQYFS IPRYGFTALQ HNEFLGQNFC
     <------------------>
          TM 2
          Glyc
601  PGLNATGNNP CNYATCTGEE YLVKQGIDLS PWGLWKNHVA LACMIVIFLT
                                    <------------------
                                              TM 3

651  IAYLKLLFLK KYS
     ------>
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FIG. 2A

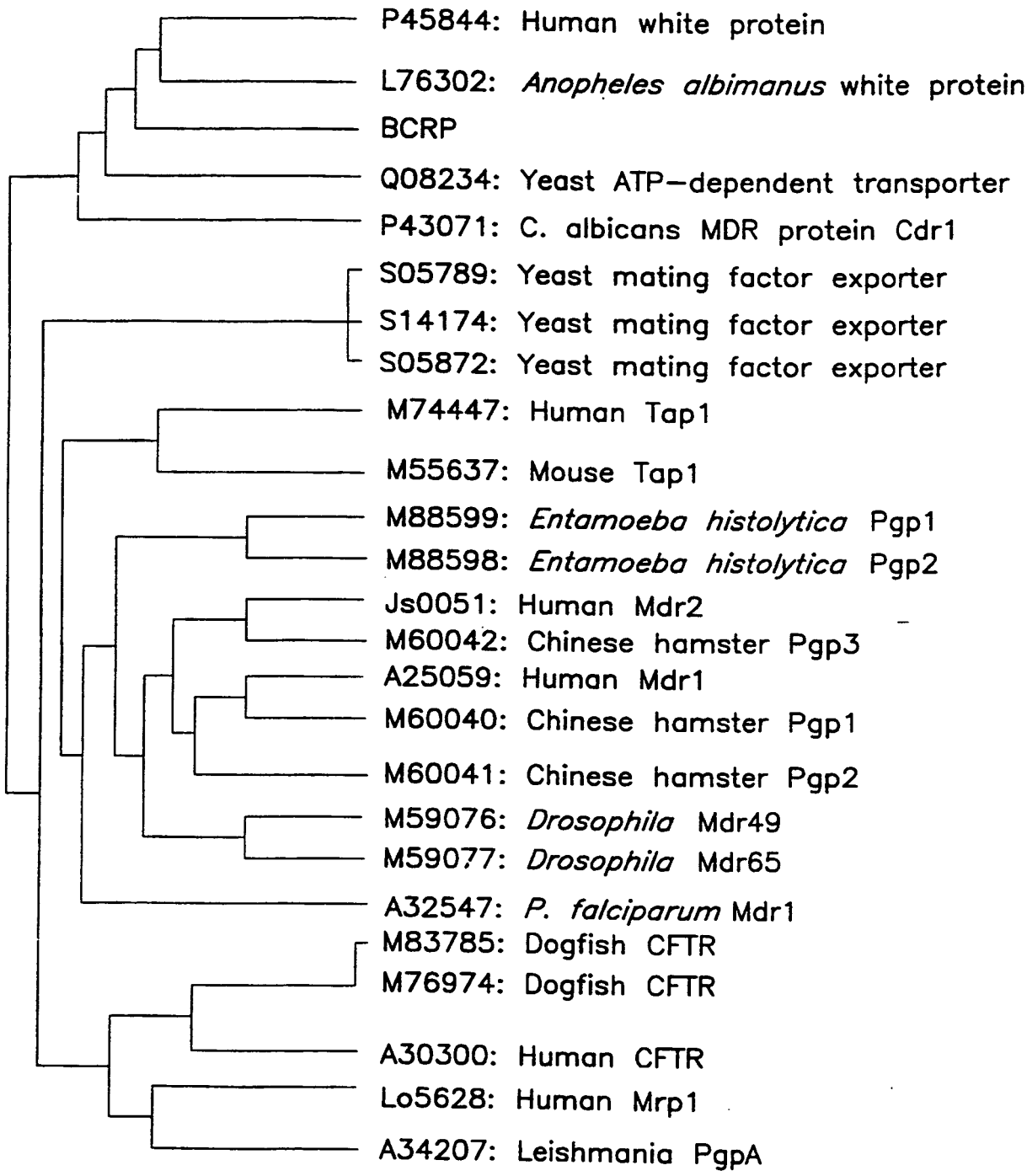

FIG. 2B

```
  1   GGGAGGAGGC AGCCTGTGGA GGAACTGGGT AGGATTTAGG AACGCACCGT
 51   GCACATGCTT GGTGGTCTTG TTAAGTGGAA ACTGCTGCTT TAGAGTTTGT
101   TTGGAAGGTC CGGGTGACTC ATCCCAACAT TTACATCCTT AATTGTTAAA
151   GCGCTGCCTC CGAGCGCACG CATCCTGAGA TCCTGAGCCT TTGGTTAAGA
201   CCGAGCTCTA TTAAGCTGAA AAGATAAAAA CTCTCCAGAT GTCTTCCAGT
251   AATGTCGAAG TTTTTATCCC AGTGTCACAA GGAAACACCA ATGGCTTCCC
301   CGCGACAGCT TCCAATGACC TGAAGGCATT TACTGAAGGA GCTGTGTTAA
351   GTTTTCATAA CATCTGCTAT CGAGTAAAAC TGAAGAGTGG CTTTCTACCT
401   TGTCGAAAAC CAGTTGAGAA AGAAATATTA TCGAATATCA ATGGGATCAT
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801   GAGGAGAAAG AAAAAGGACT AGTATAGGAA TGGAGCTTAT CACTGATCCT
851   TCCATCTTGT TCTTGGATGA GCCTACAACT GGCTTAGACT CAAGCACAGC
```

FIG. 2C-1

```
 901    AAATGCTGTC CTTTTGCTCC TGAAAAGGAT GTCTAAGCAG GGACGAACAA
 951    TCATCTTCTC CATTCATCAG CCTCGATATT CCATCTTCAA GTTCTTTGAT
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1051    GGAGGCCTTG GGATACTTTG AATCAGCTGG TTATCACTGT GAGGCCTATA
1101    ATAACCCTGC AGACTTCTTC TTGGACATCA TTAATGGAGA TTCCACTGCT
1151    GTGGCATTAA ACAGAGAAGA AGACTTTAAA GCCACAGAGA TCATAGAGCC
1201    TTCCAAGCAG GATAAGCCAC TCATAGAAAA ATTAGCGGAG ATTTATGTCA
1251    ACTCCTCCTT CTACAAAGAG ACAAAAGCTG AATTACATCA ACTTTCCGGG
1301    GGTGAGAAGA AGAAGAAGAT CACGGTCTTC AAGGAGATCA GCTACACCAC
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1401    TGCTGGGTAA TCCCCAGGCC TCTATAGCTC AGATCATTGT CACAGTCGTA
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1501    TGGAATCCAG AACAGAGCTG GGGTTCTCTT CTTCCTGACG ACCAACCAGT
1551    GTTTCAGCAG TGTTTCAGCC GTGGAACTCT TTGTGGTAGA GAAGAAGCTC
1601    TTCATACATG AATACATCAG CGGATACTAC AGAGTGTCAT CTTATTTCCT
1651    TGGAAAACTG TTATCTGATT TATTACCCAT GACGATGTTA CCAAGTATTA
                                5' 1727          1744
1701    TATTTACCTG TATAGTGTAC TTCATGTTAG GATTGAAGCC AAAGGCAGAT
                                5' PCR PRIMER (SENSE)
1751    GCCTTCTTCG TTATGATGTT TACCCTTATG ATGGTGGCTT ATTCAGCCAG
1801    TTCCATGGCA CTGGCCATAG CAGCAGGTCA GAGTGTGGTT TCTGTAGCAA
```

FIG. 2C-2

```
1851    CACTTCTCAT GACCATCTGT TTTGTGTTTA TGATGATTTT TTCAGGTCTG
1901    TTGGTCAATC TCACAACCAT TGCATCTTGG CTGTCATGGC TTCAGTACTT
1951    CAGCATTCCA CGATATGGAT TTACGGCTTT GCAGCATAAT GAATTTTTGG
2001    GACAAAACTT CTGCCCAGGA CTCAATGCAA CAGGAAACAA TCCTTGTAAC
2051    TATGCAACAT GTACTGGCGA AGAATATTTG GTAAAGCAGG GCATCGATCT
2101    CTCACCCTGG GGCTTGTGGA AGAATCACGT GGCCTTGGCT TGTATGATTG
              2152                    2172
2151    TTATTTTCCT CACAATTGCC TACCTGAAAT TGTTATTTCT TAAAAAATAT
                                      5'
2201    TCTTAAATTT CCCCTTAATT CAGTATGATT TATCCTCACA TAAAAAAGAA
2251    GCACTTTGAT TGAAGTATTC AATCAAGTTT TTTTGTTGTT TTCTGTTCCC
2301    TTGCCATCAC ACTGTTGCAC AGCAGCAATT GTTTTAAAGA GATACATTTT
2351    TAGAAATCAC AACAAACTGA ATTAAACATG AAAGAACCCA AAAAAAAAGA
2401    TATCACTCAG CATAATGA
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FIG. 2C-3

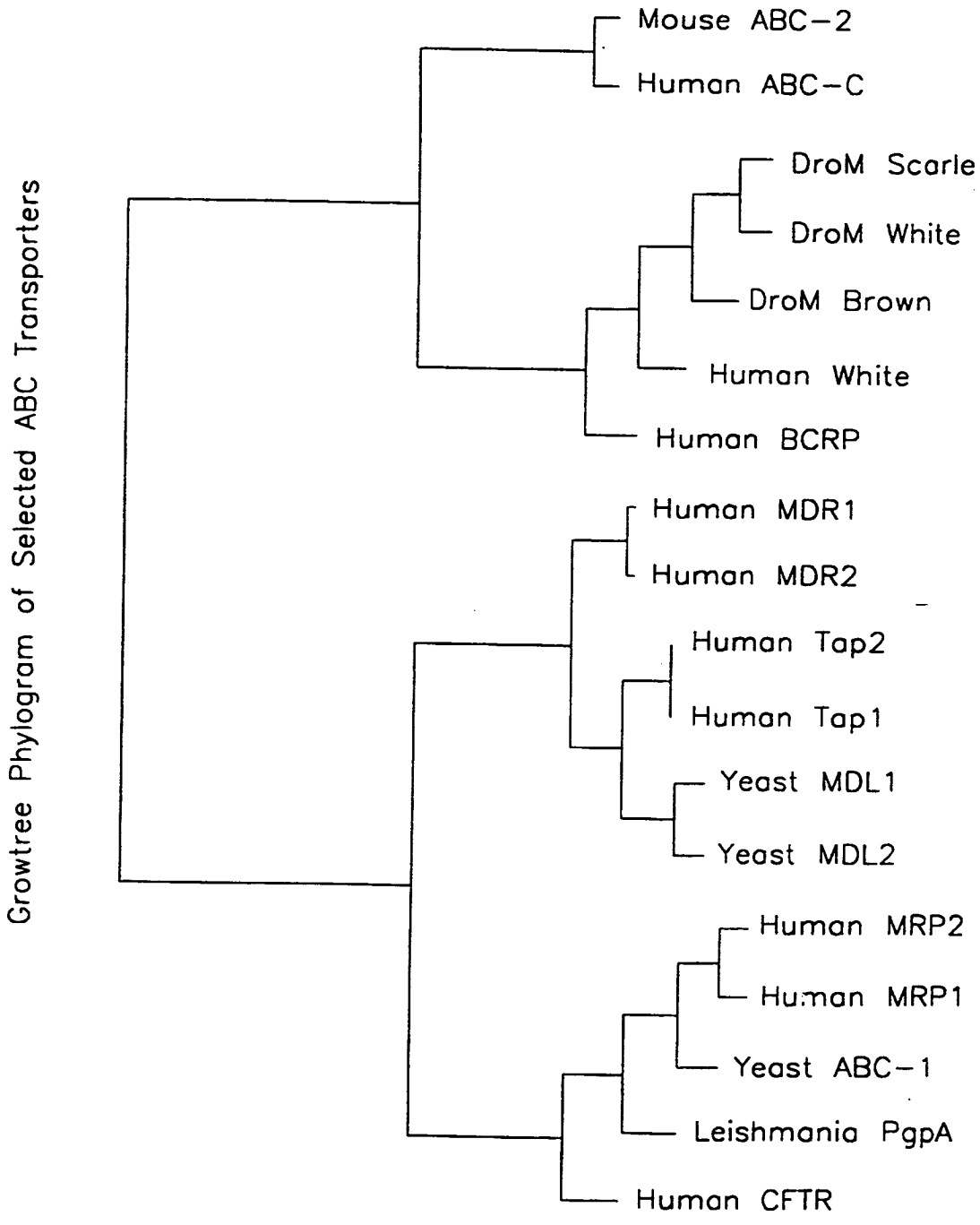


FIG. 2D

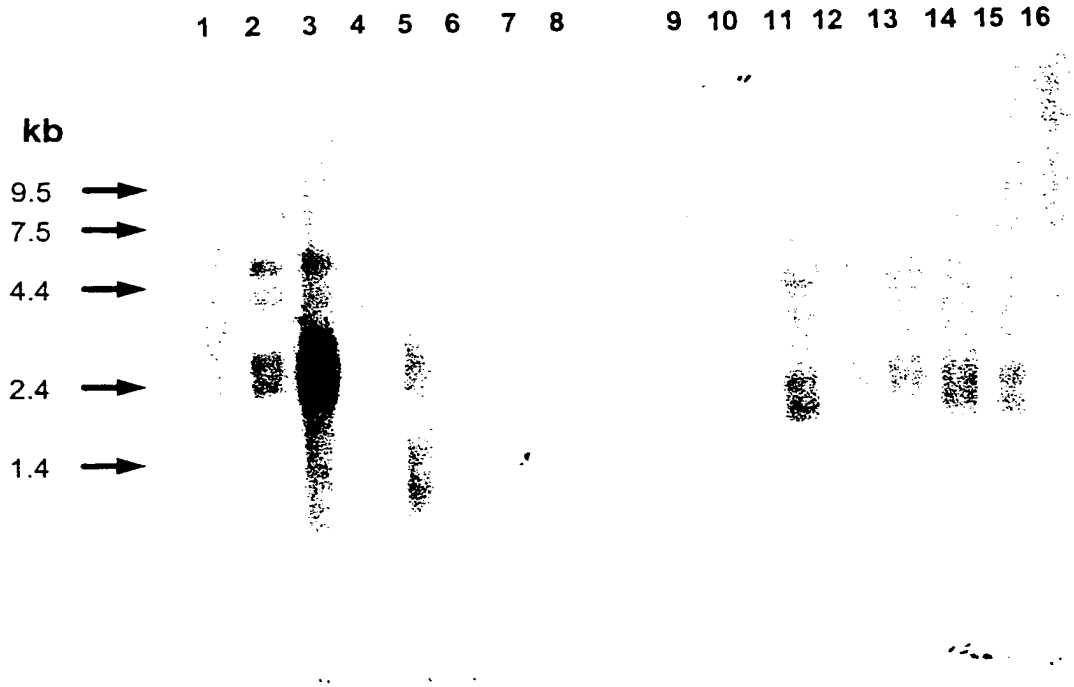


FIG. 3

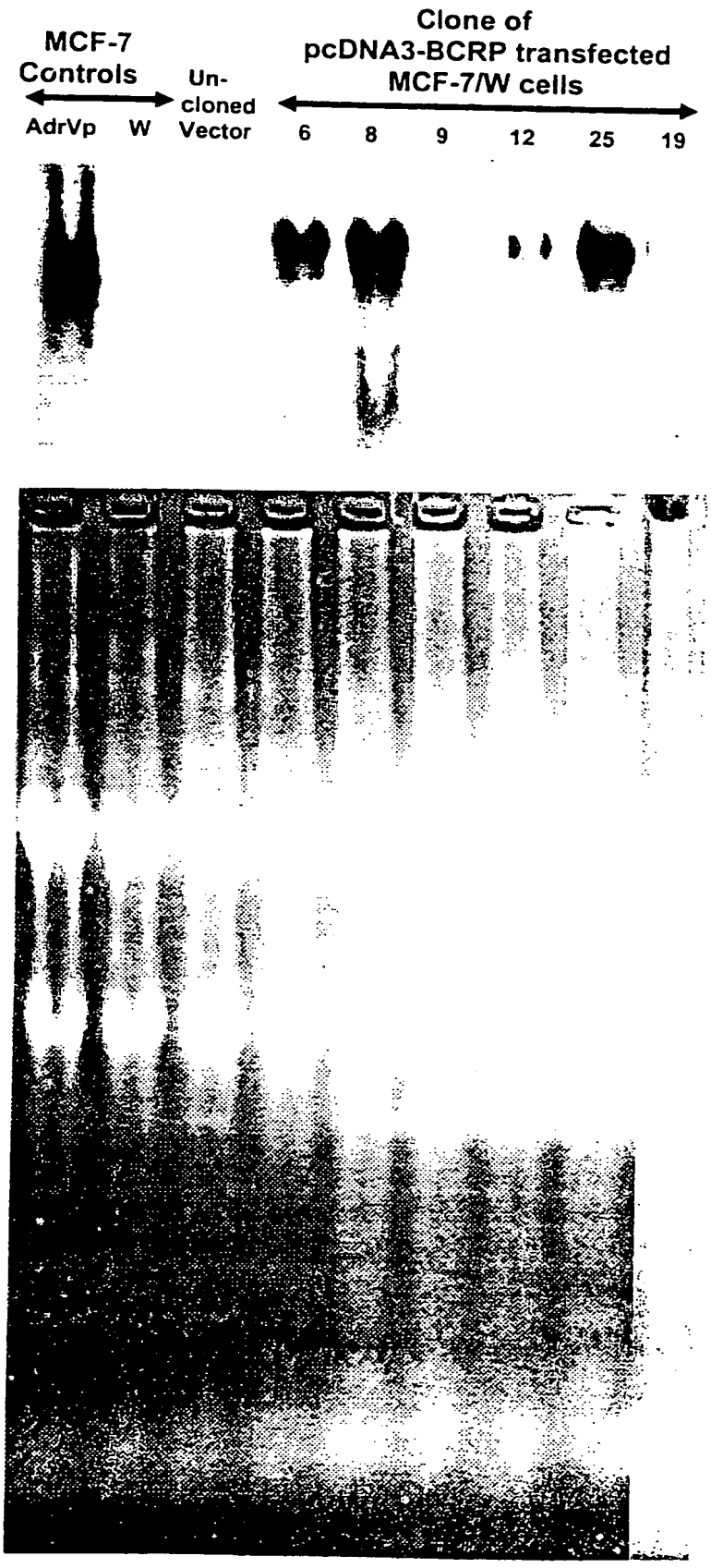


FIG. 4A

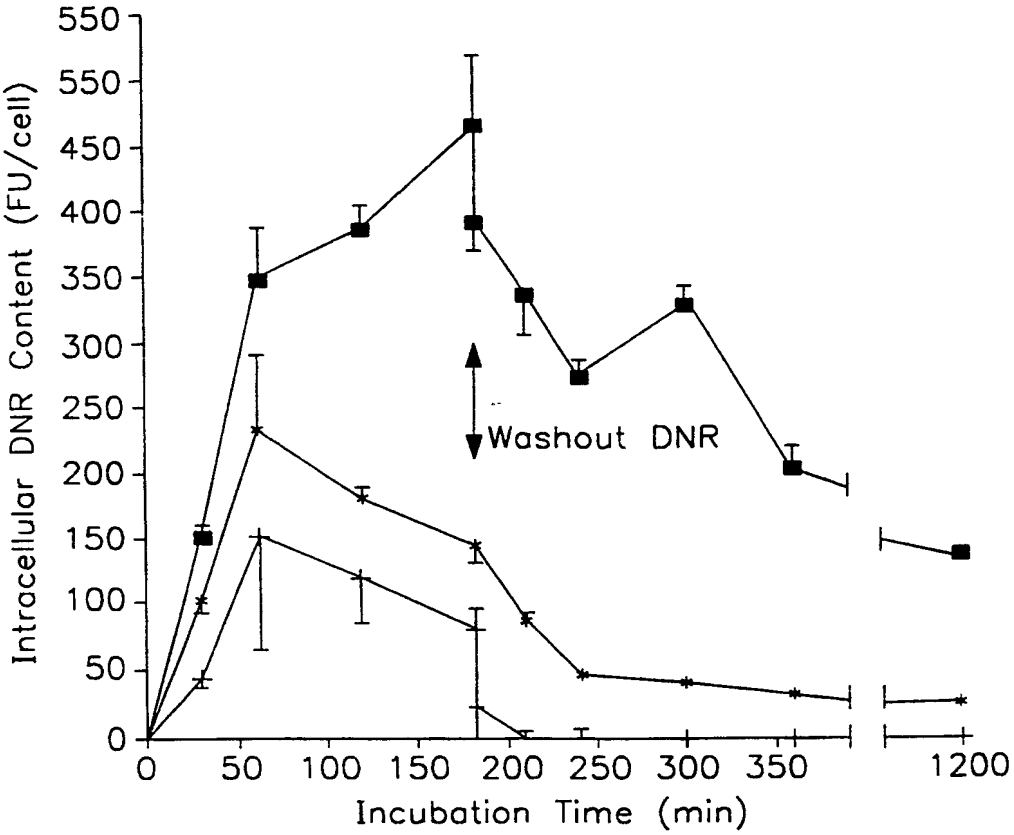


FIG. 4B

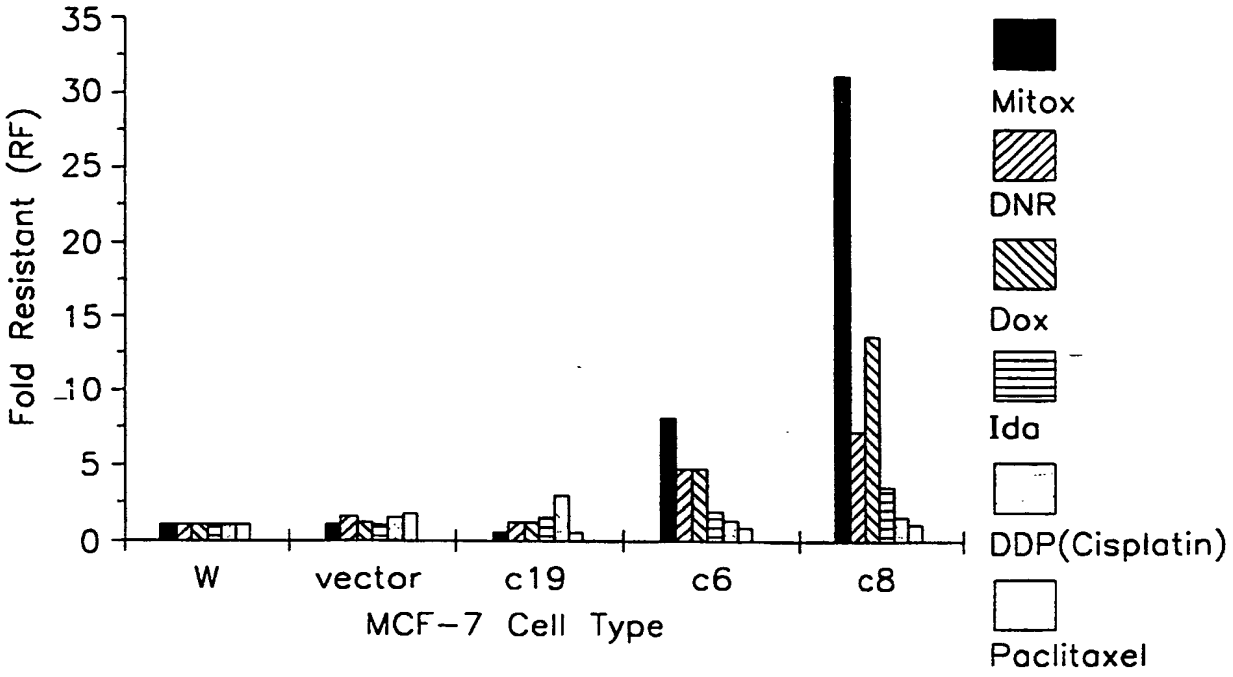


FIG. 4C

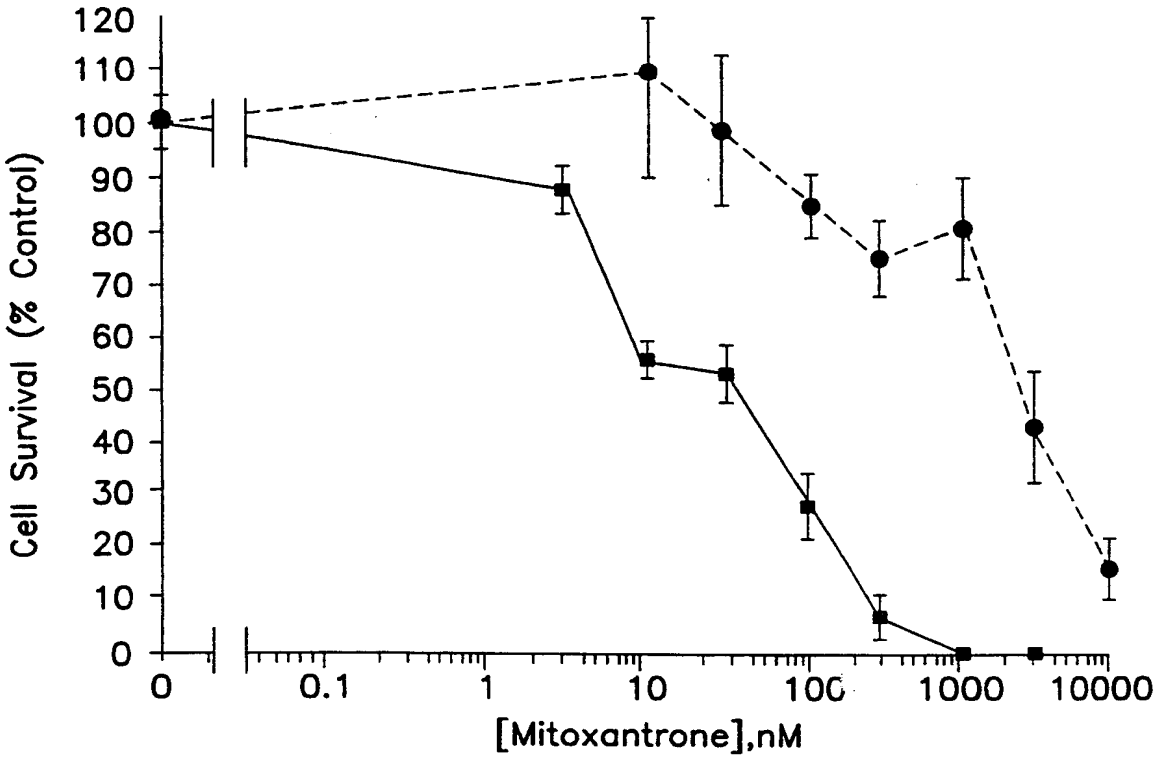


FIG. 4D-1

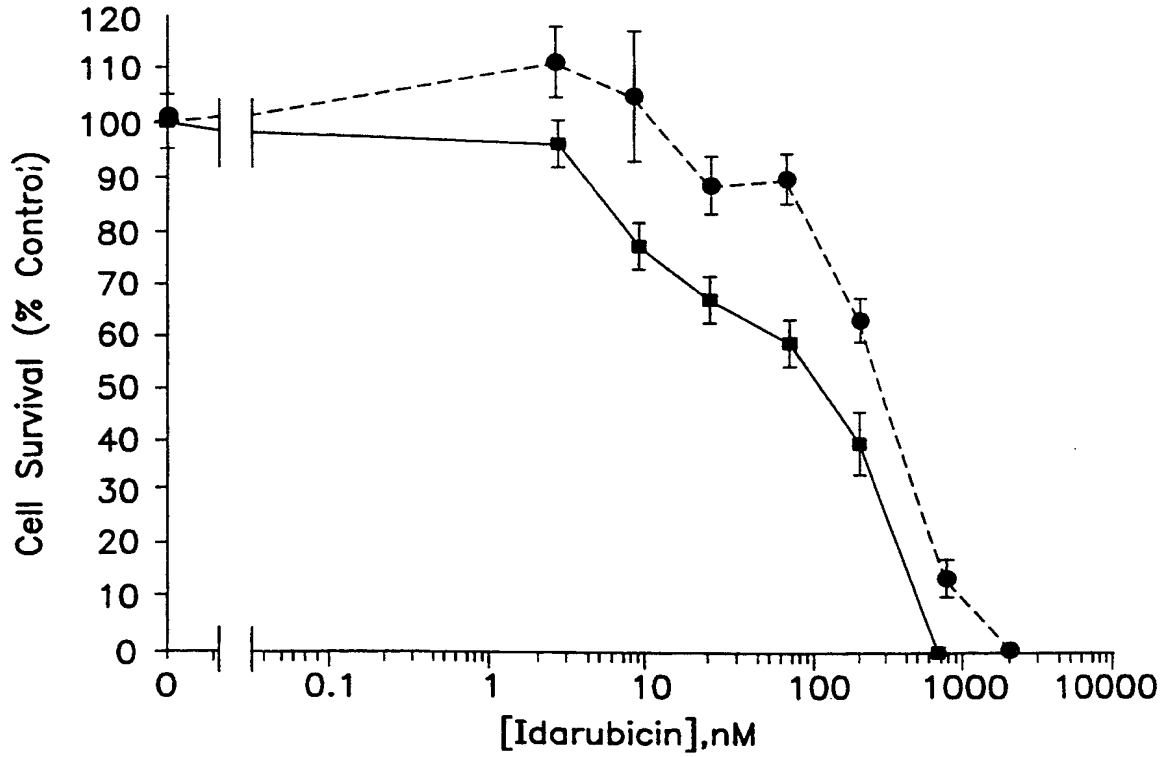


FIG. 4D-2

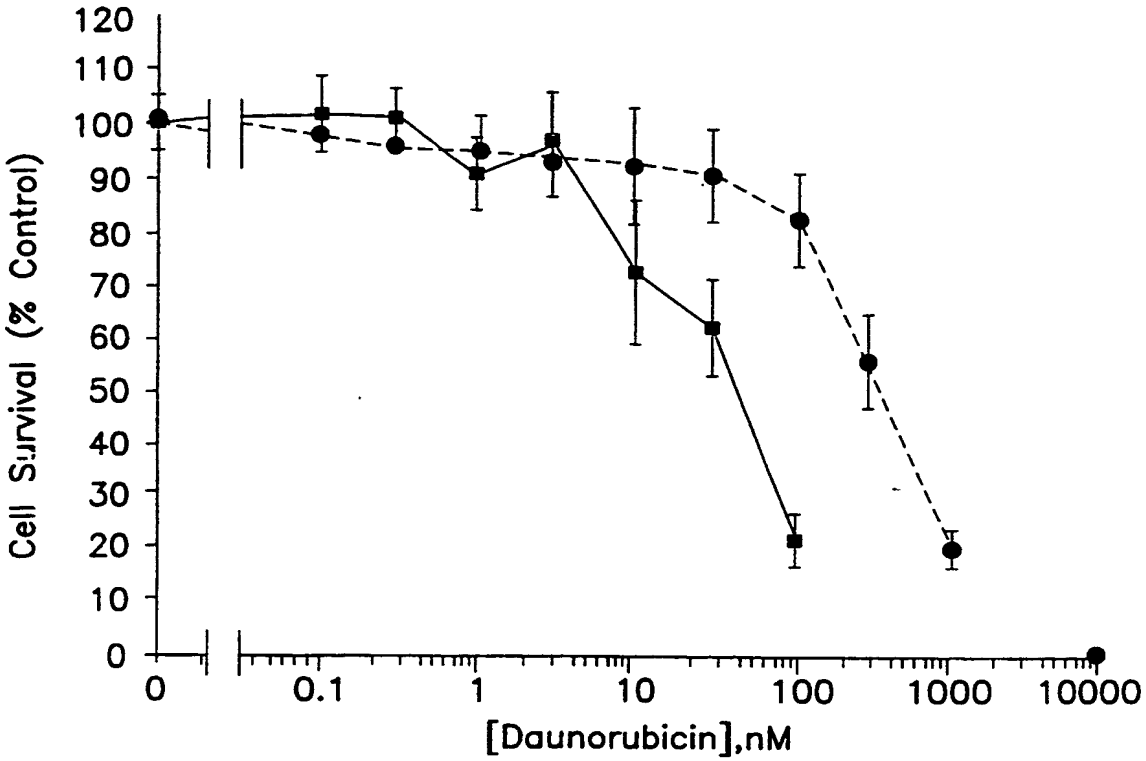


FIG. 4D-3

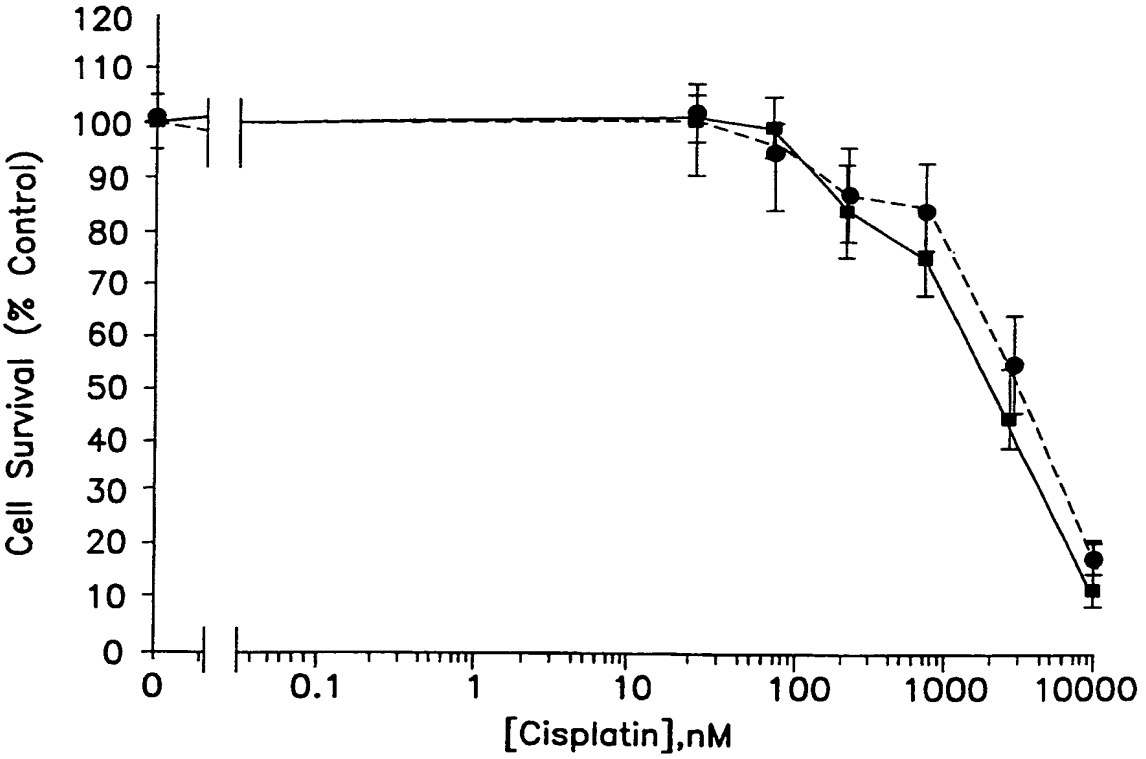


FIG. 4D-4

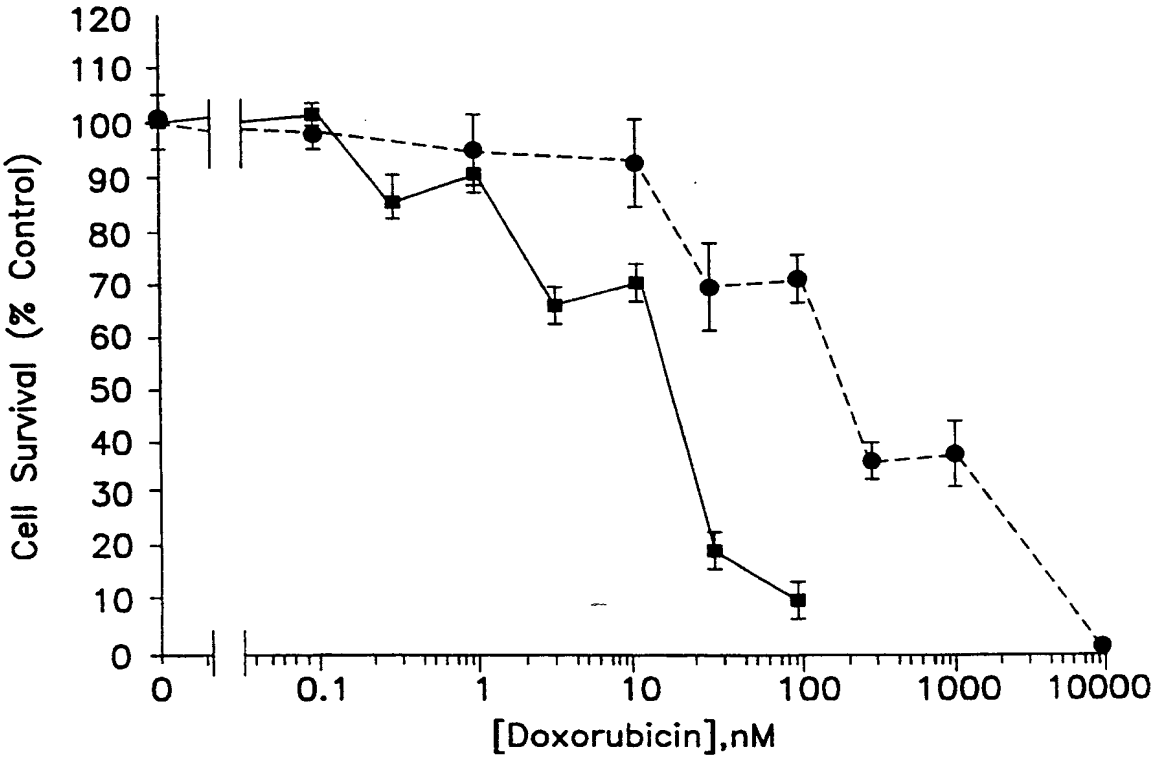


FIG. 4D-5

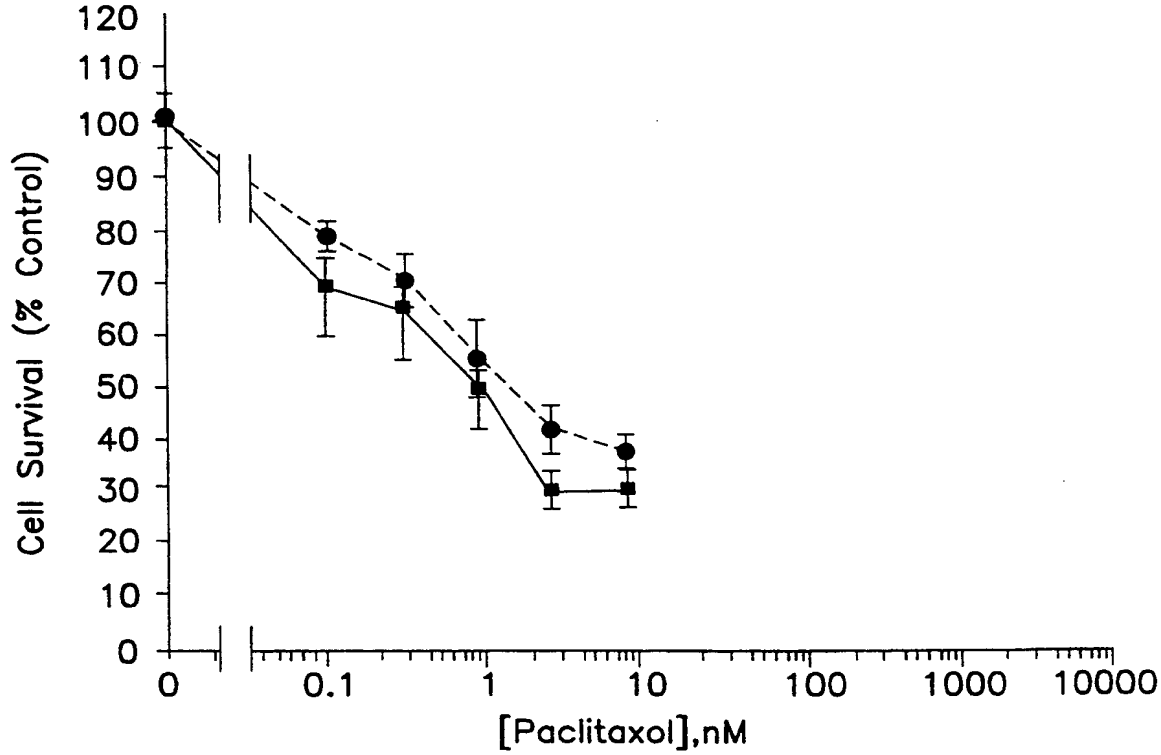


FIG. 4D-6

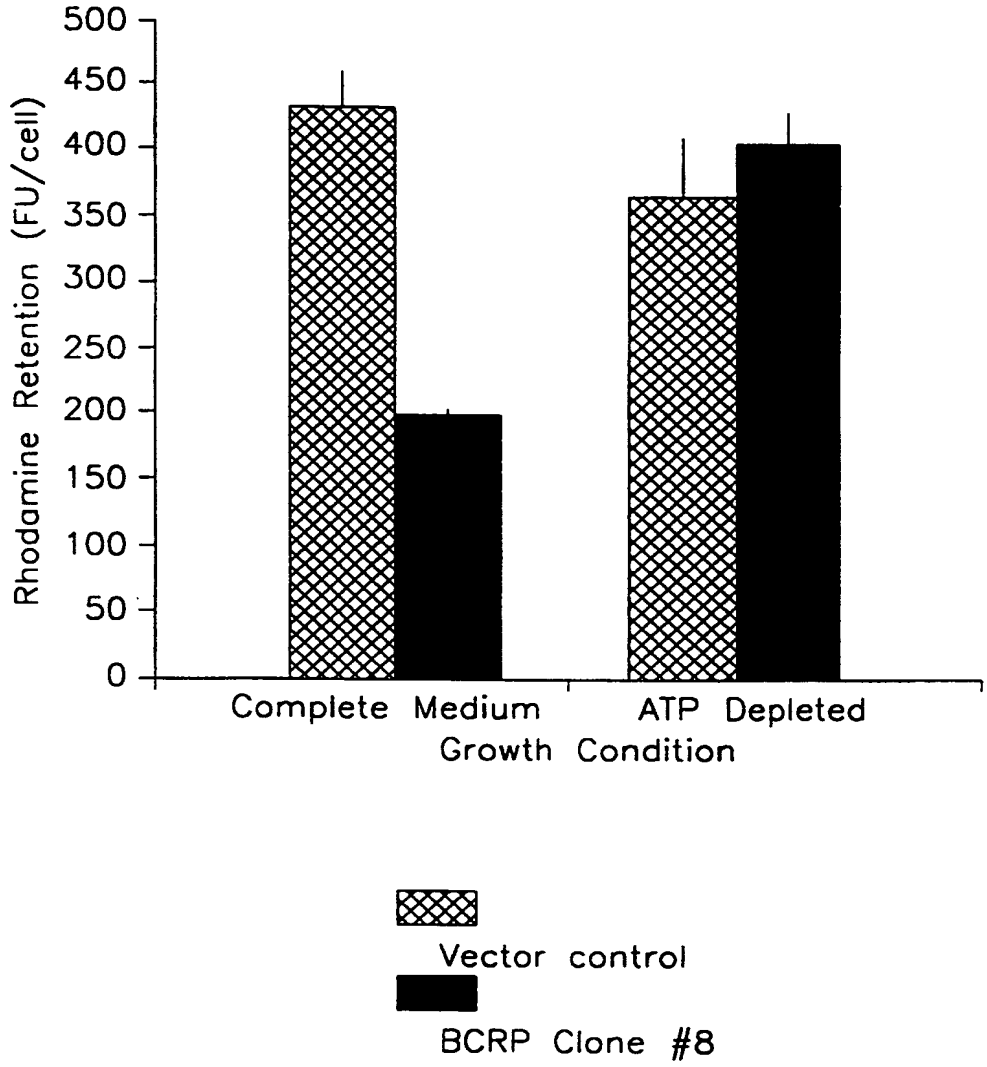


FIG. 4E

LC50, nM

Cell Line	Mitozantrons		Daunorubicin		Dexprubicin		Idarubicin		CiaPlatin		Paclitaxel	
	LC50	RF	LC50	RF	LC50	RF	LC50	RF	LC50	RF	LC50	RF
MCF-7/W	48	1.0	47	1.0	57	1.0	75	1.0	2,367	1.0	1.9	1.0
MCF-7/pcDNA3	54	1.1	72	1.5	66	1.2	126	1.7	3,525	1.5	3.0	1.6
MCF-7/BCRPc19	21	0.4	54	1.1	67	1.2	107	1.4	8,950	2.9	0.8	0.4
MCF-7/BCRPall	393**	8.2	218**	4.5	254	5.2	140	1.8	3,080	1.3	1.4	0.7
MCF-7/BCRPcS	1,495**	31.2	328**	7.0	768*	9.2	285	3.5	3,700	1.6	1.8	0.9
MCF-7/AdrVp	180,000**	3333	1667**	35.5	8650**	175.0	70	0.8	4,700	2.01	2.8	1.5

* = differs significently from MCF-7/W or MCF-7/pcDNA3, p <0.05 (Student's t test)
** = differs significently from MCF-7/W or MCF-7/pcDNA3, p <0.01 (Student's t test)

FIG. 5

Expression of Human *w* gene in MCF-7 Cells, Detected by RT-PCR

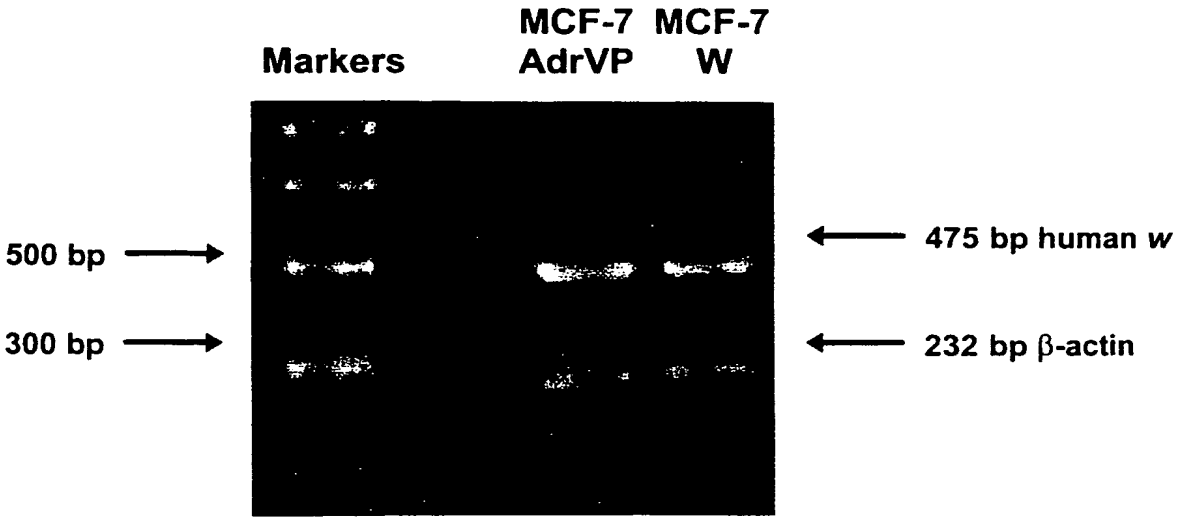


FIG. 6

RT-PCR detection of BCRP mRNA expression in MCF-7/W cells or Blast Cells from Patients with Acute Myeloid Leukemia

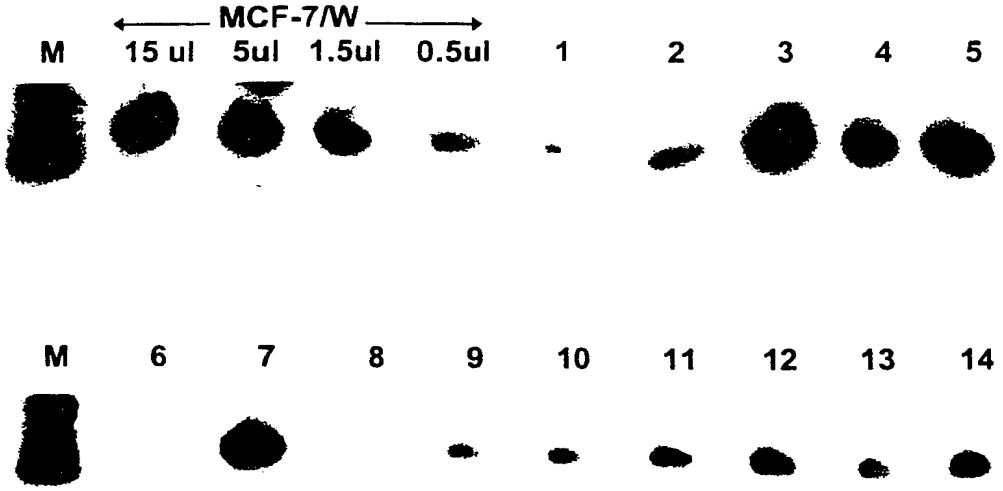


FIG. 7

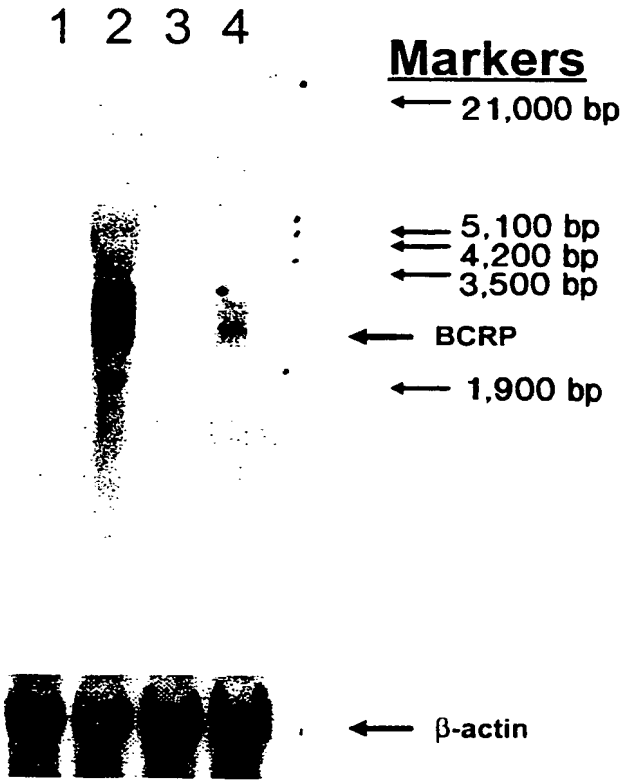


FIG. 8A

Key:

1. S1/M1-3.2
2. S1/W
3. MCF-7/W
4. MCF-7/MX$_{R}$
5. MCF-7/MX$_{RS250}$
6. MCF-7/MX$_{RS600}$
7. MCF-7/VP (MRP +)
8. MCF-7/Adr (Pgp +)
9. MCF-7/MTX (DHFR +)
10. MCF-7/AdrVp1000 (BCRP +)

FIG. 8B

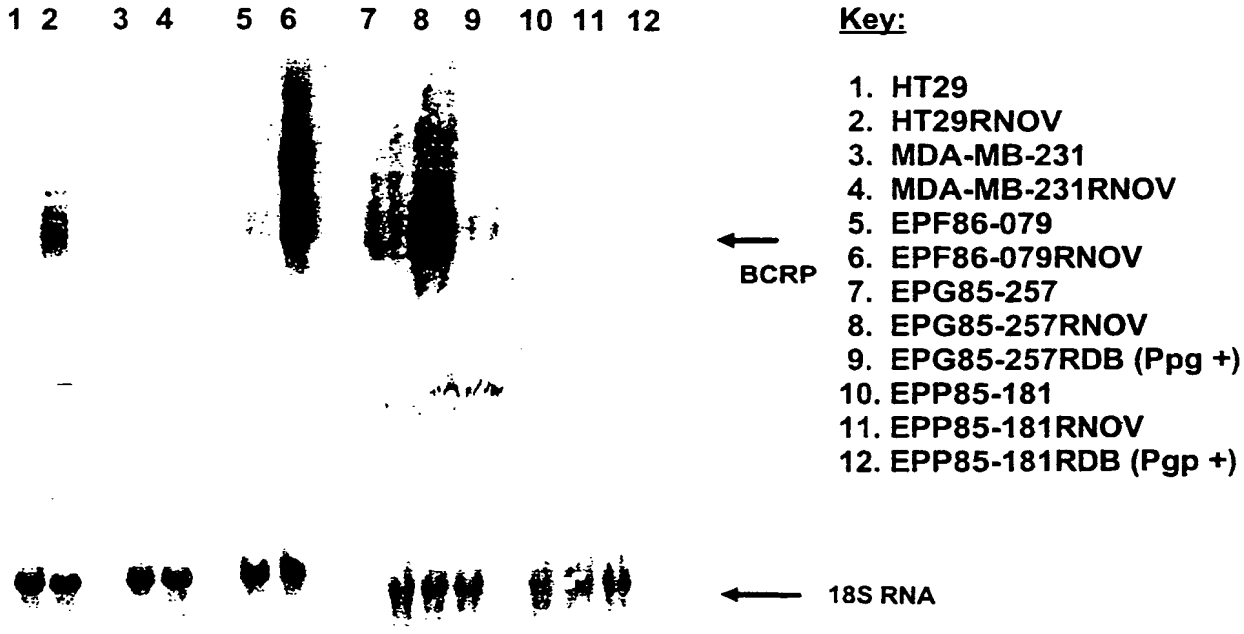
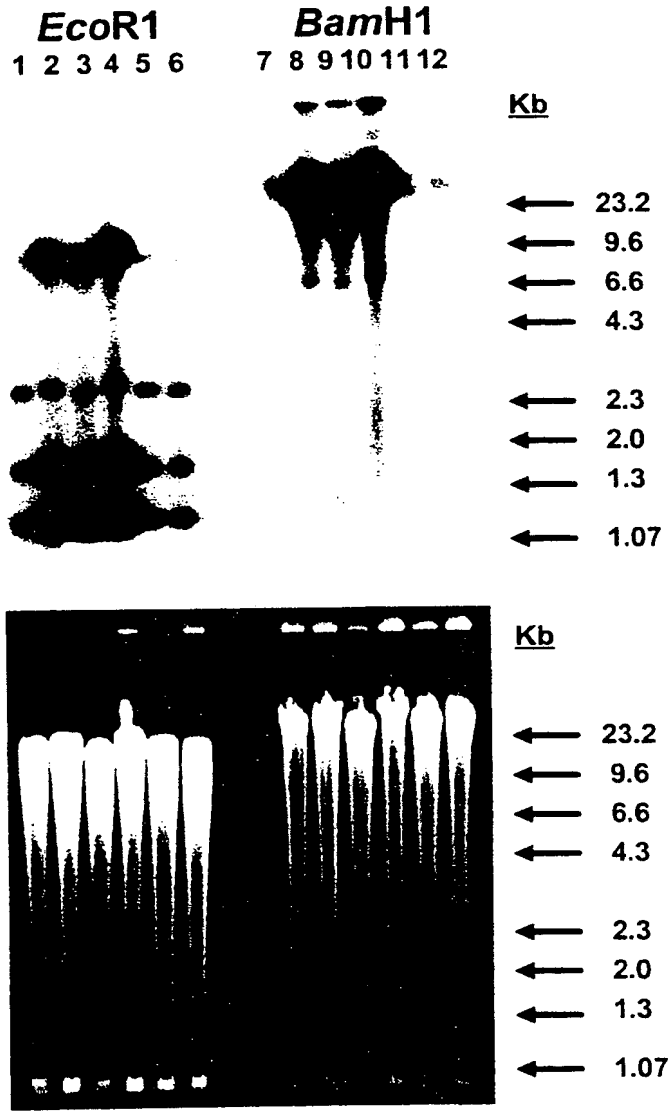


FIG. 8C



Key:

1, 7. MCF-7/W

2, 8. MCF-7/MX_{PR}

3, 9. MCF-7/MX_{RS250}

4, 10. MCF-7/MX_{RS600}

5, 11. MCF-7/VP (MRP+)

6, 12. MCF-7/MTX (DHFR+)

FIG. 9

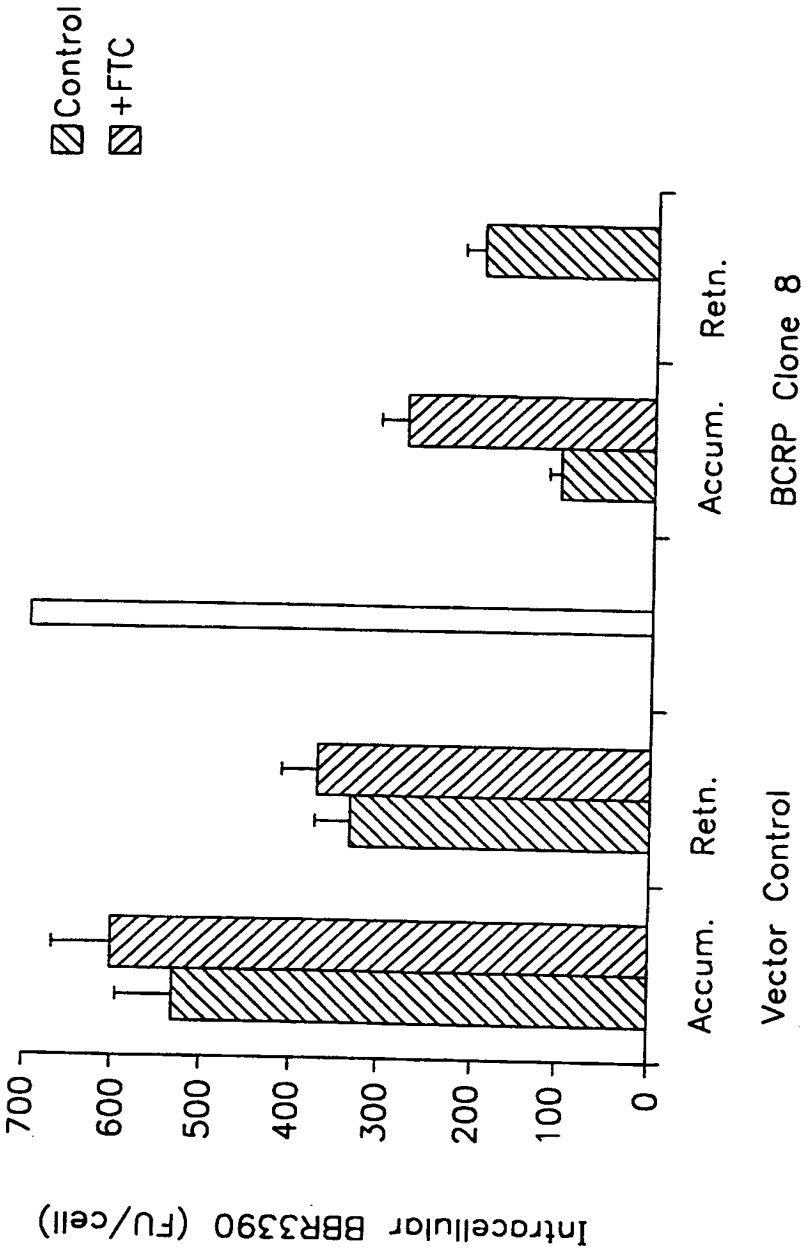


FIG. 10

SEQUENCE LISTING

<110> Doyle, L. Austin
Abruzzo, Lynne V.
Ross, Douglas D.

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<130> Ross UMb conversion

<140> 011-005
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<150> 60/073763
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Lys Ser Gly Phe Leu Pro Cys Arg Lys Pro Val Glu Lys Glu Ile Leu
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Ser Asn Ile Asn Gly Ile Met Lys Pro Gly Leu Asn Ala Ile Leu Gly
65 70 75 80

Pro Thr Gly Gly Gly Lys Ser Ser Leu Leu Asp Val Leu Ala Ala Arg
85 90 95

Lys Asp Pro Ser Gly Leu Ser Gly Asp Val Leu Ile Asn Gly Ala Pro
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    130                 135                 140

Ala Leu Arg Leu Ala Thr Thr Met Thr Asn His Glu Lys Asn Glu Arg
145                 150                 155                 160

Ile Asn Arg Val Ile Gln Glu Leu Gly Leu Asp Lys Val Ala Asp Ser
                165                 170                 175

Lys Val Gly Thr Gln Phe Ile Arg Gly Val Ser Gly Gly Glu Arg Lys
            180                 185                 190

Arg Thr Ser Ile Gly Met Glu Leu Ile Thr Asp Pro Ser Ile Leu Phe
        195                 200                 205

Leu Asp Glu Pro Thr Thr Gly Leu Asp Ser Ser Thr Ala Asn Ala Val
    210                 215                 220

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Ser Ile His Gln Pro Arg Tyr Ser Ile Phe Lys Leu Phe Asp Ser Leu
                245                 250                 255

Thr Leu Leu Ala Ser Gly Arg Leu Met Phe His Gly Pro Ala Gln Glu
            260                 265                 270

Ala Leu Gly Tyr Phe Glu Ser Ala Gly Tyr His Cys Glu Ala Tyr Asn
        275                 280                 285

Asn Pro Ala Asp Phe Phe Leu Asp Ile Ile Asn Gly Asp Ser Thr Ala
    290                 295                 300

Val Ala Leu Asn Arg Glu Glu Asp Phe Lys Ala Thr Glu Ile Ile Glu
305                 310                 315                 320

Pro Ser Lys Gln Asp Lys Pro Leu Ile Glu Lys Leu Ala Glu Ile Tyr
                325                 330                 335

Val Asn Ser Ser Phe Tyr Lys Glu Thr Lys Ala Glu Leu His Gln Leu
            340                 345                 350

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Val Thr Val Val Leu Gly Leu Val Ile Gly Ala Ile Tyr Phe Gly Leu
                405                 410                 415

Lys Asn Asp Ser Thr Gly Ile Gln Asn Arg Ala Gly Val Leu Phe Phe
            420                 425                 430

Leu Thr Thr Asn Gln Cys Phe Ser Ser Val Ser Ala Val Glu Leu Phe
        435                 440                 445

Val Val Glu Lys Lys Leu Phe Ile His Glu Tyr Ile Ser Gly Tyr Tyr
    450                 455                 460

Arg Val Ser Ser Tyr Phe Leu Gly Lys Leu Leu Ser Asp Leu Leu Pro
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Leu Gly Leu Lys Pro Lys Ala Asp Ala Phe Phe Val Met Met Phe Thr
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        515                 520                 525

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    530                 535                 540

Phe Val Phe Met Met Ile Phe Ser Gly Leu Leu Val Asn Leu Thr Thr
545                 550                 555                 560

Ile Ala Ser Trp Leu Ser Trp Leu Gln Tyr Phe Ser Ile Pro Arg Tyr
                565                 570                 575

Gly Phe Thr Ala Leu Gln His Asn Glu Phe Leu Gly Gln Asn Phe Cys
            580                 585                 590

Pro Gly Leu Asn Ala Thr Gly Asn Asn Pro Cys Asn Tyr Ala Thr Cys
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    610                 615                 620
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gtatgattta tcctcacata aaaaagaagc actttgattg aagtattcaa tcaagttttt 660
ttgttgtttt ctgttccctt gccatcacac tgttgcacag cagcaattgt tttaaagaga 720
tacattttta gaaatcacaa caaactgaat taaacatgaa agaacccaaa aaaaaagata 780
tcactcagca taatg                                                  795
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02577

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 14/00, 16/00; C07H 21/04; A61K 48/00, 39/395; G01N 33/53
US CL :530/350; 387.1; 536/23.1, 24.5; 514/44; 435/7.1; 424/130.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 387.1; 536/23.1, 24.5; 514/44; 435/7.1; 424/130.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS MEDLINE EMBASE BIOSIS CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	DOYLE et al. A Multidrug Resistance Transporter from Human MCF-7 Breast Cancer Cells. Pro. Natl. Sci. USA. December 1998, Vol 95, pages 15665-15670. See entire document.	1-19
X,P	RABINDRAN et al. Reversal of a Novel Multidrug Resistance Mechanism in Human colon Carcinoma Cells by Fumitremorgin C. Cancer Research. 15 December 1998, Vol. 58, No. 24, pages 5850-5858. See entire document.	20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 APRIL 1999	Date of mailing of the international search report 11 MAY 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer LIN SUN-HOFFMAN Telephone No. (703) 308-0196 JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02577

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database CAPLUS on STN, AN 1998:808188. ZHU et al. 'Pharmacological Activities of Aspergillus Fumigatus Products and Prospects as New Anti-Tumor Drugs'. Zhongguo Yaoxue Zazhi (Beijing). 1998, Vol 33, No 11, pages 645-648. See abstract only.	20